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The Journal of Biochemistry, Vol. 37, No. 4.

METABOLISM OF TYROSINE

II. HOMOGENTISICASE*

By

MASAMI SUDA and YOSHIRO TAKEDA

(*Department of Bacteriochemistry, Research Institute for Microbial Diseases, Osaka University, Osaka*)

(Received for publication, June 7, 1950)

As reported in the preceeding communication (1), cell free enzyme, catalyzing oxidative breakdown of homogentisic acid, has been isolated from the dry preparation of bacterial cells. Present communication is concerned with the isolation and partial purification of the same enzyme from rabbit liver.

Besides the ordinary co-enzyme, homogentisicase requires another active principle, ferrous ion. We analyzed further the mode of enzyme action as described below.

EXPERIMENTAL.

Purification of the Enzyme—100 g. of rabbit livers are ground well with 125 ml. of $M/25$ phosphate buffer (pH 7.0) and 50 g. of quartz sand; centrifugation; 100 ml. of the supernatant are added with 10 ml. of $M/5$ acetate buffer (pH 4.0); centrifugation; the pH of the supernatant is adjusted to 7.0 with $2N$ NaOH; and 1/10 volume of kaolin is added; centrifugation; the supernatant is heated at 55° for 5 minutes; centrifugation; the supernatant is fractionated with 0.5–0.7 saturated ammonium sulfate; the precipitate formed is dissolved in 20 ml. of $M/10$ phosphate buffer (pH 7.0) and then refractionated with addition of ammonium sulfate as above; the precipitate obtained is dissolved in 10 ml. of the same buffer. This enzyme solution is used in the following experiments.

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* The compendium of this article was announced at the Annual Meeting of the Society of Japanese Biochemists in April 1949 at Kyoto University and at the Symposium on Enzyme Chemistry in October 1949 at Tokyo University.

Some Properties of the Enzyme—In the course of above preparation, the enzyme activity dropped suddenly at the stage of fractionation with $(NH_4)_2SO_4$. Upon addition of $10^{-3}M$ ferrous ion, however, the inactivated liquid recovered most of the original activity.

When the final enzyme solution is dialyzed against destilled water for 24 hours, the inner liquid is not activated with ferrous ion alone, but activated with further addition of its dialysate. It is possible to substitute the dialysate with heated extract of the liver. (Fig. 1).

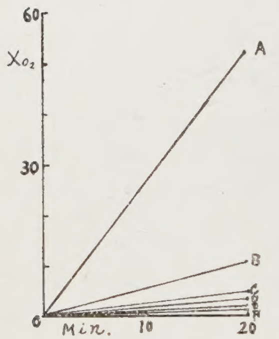


Fig. 1. Components of the purified Homogentisicase.

A, apo-enzyme+boiled extract of rabbit liver+$FeSO_4$.

B, apo-enzyme+boiled extract.

C, apo-enzyme+$FeSO_4$.

D, apo-enzyme alone.

E, boiled extract+$FeSO_4$.

F, boiled extract alone.

Substrate: 0.2 ml. of $M/100$ homogentisic acid. Final concentration of $FeSO_4$ is $10^{-3}M$ pH of each vessel is adjusted to 7.2. 0.2 ml. of 10% KOH in center well. Final volume of main compartment 2.2 ml. Temperature 30°.

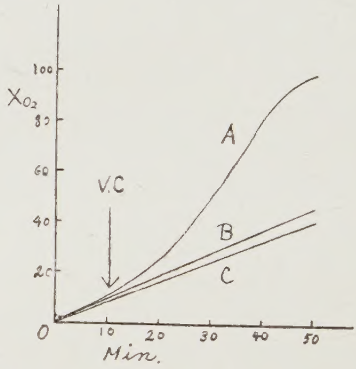


Fig. 2. The recovery of the enzyme activity by addition of ascorbic acid in the presence of ferric ion. The enzyme used is not dialyzed, but fractionated with $(NH_4)_2SO_4$. Ferric sulphate $10^{-3}M$ is added at the start. Temperature 30°.

A, ascorbic acid (2 mg. per vessel in the side arm) is tipped in the main compartment after 10 minutes.

B, enzyme+ferric sulphate.

C, enzyme+ascorbic acid.

The purified enzyme is not activated with ferric ion, but when ascorbic acid is added in order to reduce ferric ion to ferrous, the activity is regained prominently as shown in Fig. 2. 10^{-3} M $\alpha\alpha'$ dipyridyl inhibits the enzyme action observed in all the stage of purification. (Fig. 3) Other reduced metallic ions can not serve as substitutes for ferrous ion.

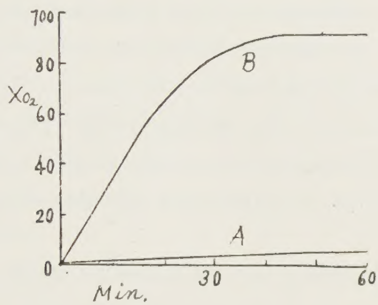
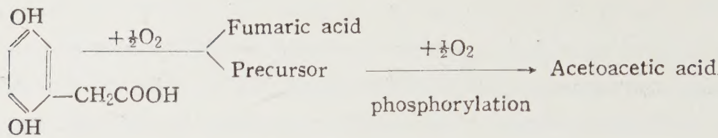


Fig. 3. Inhibition of enzyme activity by $\alpha\alpha'$-dipyridyl.
A, 1 ml. of enzyme+0.2 ml. of $M/100$ $\alpha\alpha'$-dipyridyl.
B, enzyme alone.
Substrate: 0.2 ml. of $M/50$ homogentisic acid. Final volume, 2.2 ml. Temperature 30°.

The Mode of Enzyme Action—Determination of fumaric acid and acetoacetic acid were performed manometrically as reported by Krebs *et al.* (2) and Edson (3) respectively, after the termination of the reaction. The results corresponded to 80% and 93% of the theoretical value for fumaric acid and acetoacetic acid respectively. O_2 uptake was calculated to be 2 atoms of oxygen per mole of substrate, no CO_2 release being measurable. It is important that O_2 uptake is reduced by half, one atom per mole, when $M/25$ borate buffer is used instead of phosphate buffer and that in this case no acetoacetic acid is determined while fumaric acid is detected.

Phosphate can be substituted with arsenate to give the same results, but ATP does not participate with this reaction. The mode of enzyme reaction is postulated as follows:



In this two-step reaction, we designate the enzyme which ruptures the benzene ring "homogentisicase." The precursor of acetoacetic acid is unknown at the present time, but crotonic acid has been excluded. It may be isocrotonic acid or vinyl acetic acid, but they have remains unavailable for us.

The optimum pH of the enzyme is 7.0—7.2. FAD, cytochrome C, aneurinpyrophosphate, and coenzyme I were found to be inactive as the coenzyme. Experiments to determine the latter are in progress.

Because this enzyme is not inhibited by KCN, monojodoacetate, NaF, dimedon or semicarbazide, and further by aniline, it may be assumed that benzene ring is ruptured directly with association of oxidation and without the formation of quinone substances, as previously reported in the case of pyrocatecase (4).

Homogentisicase is adsorbed with C_γ and eluted with 2% $(NH_4)_2HPO_4$ solution pH 7.6 adjusted with concentrated aqeous ammonia. A portion of coenzyme appears to be separated from the bulk of homogentisicase by this procedure.

Discussion

In this connection it should be mentioned that appearance of homogentisic acid in the urine of ascorbic acid deficient animals, which was reported by Sealock *et al.* (4) is presumably related to ferrous ion which is one of the active parts of homogentisicase. Generally speaking, it does not seem to be unnatural that ascorbic acid is required in greater amount compared with other vitamines because it plays the role in many reaction related to ferrous ion in living cells.

SUMMARY

1. From rabbit liver we have isolated and purified an enzyme, which catalyzed the oxidative breakdown of homogentisic acid with the rupture of benzene ring.

2. The enzymatic reaction proceeds in two steps. In the first benzene ring is ruptured accompanying the consumption of 1 atom of oxygen, and both fumaric acid and precursor of acetoacetic acid are produced. In the second the precursor is then converted to acetoacetic acid with uptake of 1 atom of oxygen. In this step of the reaction inorganic phosphate is required. This may suggest that phosphorylation is involved in the reaction.

3. We designate the enzyme which ruptures the benzene ring as "homogentisicase."

4. Homogentisicase consists of a dialysable co-enzyme, apo-enzyme, and ferrous ion.

5. The relation between ascorbic acid and metabolism of tyrosine was discussed.

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The Journal of Biochemistry, Vol. 37, No. 4.

ON THE TUBERCULOSTATIC ACTIONS OF VARIOUS ORGANIC COMPOUNDS.

II. FATTY ACIDS AND THEIR DERIVATIVES.

By

YOSHIAKI MIURA

(*Department of Biochemistry, Faculty of Medicine, Tokyo University, Tokyo*)

(Received for publication, June 30, 1950)

INTRODUCTION

During researches on chemotherapy of tuberculos:s, considerable work has been carried out with the fatty acids and their derivatives. From the historical view, the possibilities of anti-tubercular action of the leprocidal chaulmoogra oil itself, and of its cyclic unsaturated fatty acid constituents were also investigated; and many structurally simillar carbon-chain fatty acids, containing rings or branched chains, were synthesized (1). Many of these natural and synthetic substances were tuberculostatic in test tube. A number of them were tested in experimentally infected animals and some prolongation of life and suppression of lesions were claimed, but the matter remained in doubt; trials in human pulmonary tuberculosis also were inconclusive (2) (3). (See also Table II).

However, work in this field has received a new encouragement from the chemical analysis of lipids of the mycobacteria (4). These results and the discovery of phthioic acid stimulated the synthesis by other workers, of lipid-soluble or lipophilic substances that might be expected to penetrate the supposed waxy or fatty "envelope" of the bacillus, or to injure it in anyway, for instance through a surface tension effect. The synthesis of branched-chain fatty acids and those chemically related to natural bacillary con-

stituent seemed to offer reasonable promise of blocking some essential biosynthesis (5) (6). Thus, there seems to exist at least two ideas on the mechanism of growth inhibition: one physicochemical (lipolysis or penetration) and the other, chemical (synthesis-blocking) which guide the current work on the tuberculostatic action of fatty acids and their derivatives.

Here, the author will present the experimental results on the *in vitro* tuberculostatic effect of saturated or unsaturated fatty acids, and their derivatives such an esters, taurid, rhodanised and halogenised.

Experimental

1. *Experimental Method.*

1. Methods used for the *in vitro* assays were similary to those of employed in the previous report (7). It is necessary, however, to describe some cautions taken particulary for fatty acid derivatives.

The liquid test compounds were weighed in small capirallies of known weight and put into acetone. All test compounds were sterilized without heat, avoiding vaporisation. Some of the unsaturated fatty acids were tested as soon as they were prepared, thus avoiding oxidation.

On account of the influence of surface activity some of representatives test compounds were also assayed on agar slant culture as well as on semi-soft colloidal culture using 5% carboxy methylcellulose, but the results obtained by semi-soft and solid culture were approximately similar to those by the standard liquid culture.

The results are shown by the maximum effective dilution in mg% to suppress the surface growth of *M. tuberculosis var. Gallinaceum* in synthetic medium at the end of a week.

2. *Experimental Results.*

The results obtained are shown in the next Table.

TABLE I.

I. Saturated straight chain fatty acids.

$CH_3(CH_2)_nCOOH$

No. of C	4	6	8	10	12	14	16	18
Activity in mg%	5	5	10	10	5	5	10	>10

II. Unsaturated, Straight-chain fatty acids.

No. of C	9*	18		
Degree of Unsaturation	1	1	1	3
Configuration	trans	cis	trans	cis
Activity	>10 (α—nonenic)	0.31 (oleic)	5 (elaidic)	0.5 (linolenic)

III. Unsaturated, straight-chain fatty acids ester.

No. of C (not including C of ester part.)	9*	18
Degree of Unsaturation	1	3
Configuration	trans	cis
Activity	>10 (α—nonenic acid Me ester)	5 (linolenic acid Me ester)

IV. Branched-chain fatty acids.†

1. α—Branched chain fatty acids.

$$\begin{array}{l} CH_3(CH_2)_nCHCOOH \\ \qquad\qquad\quad | \\ \qquad\qquad\quad R \end{array}$$

No. of C atoms in main part	8	11	13	14	17	19
$R = -CH_3$	—	—	—	0.63	—	—
$R = -C_2H_5$	10	1.25	1.25	0.16	10	>10
$R = -C_6H_5$	—	—	—	0.5	—	—

* α—Nonenic acid derivatives were supplied very generously by Associated Prof. T. Asahara.

† Branched—chain fatty acids were kindly supplied by the late Prof. M. Asano and his collaborators.

2. β—Branched-chain fatty acids.

$$CH_3(CH_2)_n-\underset{\displaystyle R}{\underset{|}{CH}}-CH_2COOH$$

No. of C atoms in main part	14
R= $-CH_3$	1.25

3. γ—Branched-chain fatty acids.

$$CH_3(CH_2)_n-\underset{\displaystyle R}{\underset{|}{C}}H(CH_2)_2COOH$$

No. of C atoms in main part	14	15	16
R= $-CH_3$	2.5	—	5
R= $-C_2H_5$	—	2.5	—

4. ε—Branched-chain fatty acids.

$$CH_3(CH_2)_n\underset{\displaystyle R}{\underset{|}{C}}H(CH_2)_4COOH$$

No. of C atoms in main part	10	12	14
R= $-CH_3$	—	10	1.25
R= $-C_2H_5$	5	2.5	0.63

V. Halogen-substituted fatty acids§

No. of C	3	4	5	6	7	8	9	10	11	12
α—mono Br.	—	10	—	10	—	>10	—	2.5	—	2.5
α—mono Br. Et. ester	—	10	—	10	—	10	—	10	—	>10
ω—mono Br. Et. ester	>10	—	>10	—	—	—	5	—	—	—

VI. Rhodanized fatty acids§

No. of C	4	5	6	7	8	9	10	11	12
α-mono SCN	>10	—	>10	—	>10	—	5	—	5
ω—mono SCN	—	5	—	—	—	—	—	—	—
ω—mono SCN Et. ester	—	10	—	—	—	5	—	—	—

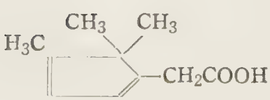
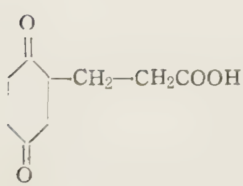
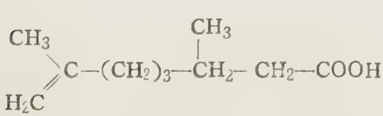
§All the compounds were supplied through the courtesy of late Dr. F. Yanase, Second Faculty of Engineering, Tokyo University.

VII. Fatty acids tauride

$CH_3(CH_2)_n{-}CONH(CH_2)_2SO_3H$

No. of C. in fatty acid portion	10	12	14	16	18
activity	>10	>10	>10	>10	>10

VIII. Miscellaneous fatty acids.

Compounds	mg%
$CH_3CO\,CH(C_2H_5)\,COOH$	10
$CH_3(CH_2)_{11}CH(CH_3)\,CO\,NH_2$	>10
$CH_3(CH_2)_7CO(CH_2)_4COOH$	>10
$CH_3(CH_2)_7C(=N.NHCONH_2).(CH_2)_4COOH$	>10
	>10
	>10
	>20

Comment

Among these 64 fatty acid derivatives examined, the most effective one is α—ethylmyristic acid and the second, α—phenyl-myristic acid. But, in animal experiment of the latter with mice, there was no evidence of suppressive effect on the development of tuberculous lesions.

Comparing the effectiveness of each series, the author noted the following general tendance.

1) There is some maximum activity along the increase of carbon atoms in the alkyl chain of fatty acids from 8 to 18, which is generally in the neighborhood of C_{14}—acid (myristic acid).

2) Comparing C_{18} saturated fatty acid to those of unsaturated fatty acids, the latter is more effective than the former. The cis-form of the acids are more effective than the trans-forms.

3) Fatty acid esters are generally less effective than the free acids.

4) In the branched-chain fatty acid series, the myristic acid derivatives, have the maximum tuberculostatic activity whenever the branched-chain groups are methyl, ethyl and phenyl or the position of the branch is α, β, γ, or ε.

5) As a branched-chain, the ethyl group is the most favorable one.

6) In the series of halogenized or rhodanized fatty acids, ω-substituted groups are more effective than the α-substituted.

7) Fatty acids taurid and other miscellaneous fatty acids, including *l*-rhodic acid, are not effective.

From the results of these experiments, conclusion will be drawn that, with increase in the length of alkyl chain (the straight or branched-chain fatty acids), the substance rapidly becomes more effective until a maximum is reached in the 14-carbon chain, and then again diminishes in effectiveness. Since the effective acids differ from each other rather widely in chemical structure, it was concluded that their bacteriostatic effectiveness was due to a

certain combination of physical properties common to all of these acids, rather than to any specific chemical structure. However, the fact that some of the branched-chain fatty acids are more effective than others would suggest some blocking effects on a specific enzyme system of tubercle bacilli. Nevertheless the grade of their antitubercular activities is increasing continuously to the maximum according to the general law and there is no specific powerful, action of some exceptionnally effective compound. Thus the author presumed that, there is no specific blocking effect of enzyme in branched-chain fatty acids but only their physico-chemical properties are more favorable than other fatty acids to suppress the growth of bacilli under experimental conditions.

A study of the physical properties of the fatty acids should be undertaken to determine whether any correlation could be found between one or more of these properties and bacteriostatic effectiveness of the acids. Among the physical properties, the surface tension is not the sole moment as assumed by Roger Adams, *et al.*, in 1932 (8). The solubility of these fatty acids to lipoids of the cell might be also important (9) (10).

Table II

Fatty acids and derivatives	in vitro	Exp. animal	Man
Chaulmoogra derivatives (3) (5) (16) (17) (18)	+	+	?
Synthetic alicyclic acids (3)	+	?	0
Branched-chain fatty acids and derivatives (1) (5)	+	0	0
Dialkyl succinic acid derivatives (20)	+	0	0
Unsaturated, long-chain fatty acids (21) (22)	+	0	0
Saturated, long-chain fatty acids (5) (22) (23)	+	±	?
Fatty acid-dye compounds (24)	?	±	0

+ effective ± doubtful ? not known 0 not tested

Moreover, recent study on surface active substances have clarified that the behavior of the mycobacterium to surface active

substances is much affected by the coexistance of albumin in the medium (11–15).

Although innumerable investigations on various fatty acids have been reported, dating back to the early days of chemotherapy (see in Table II, in which some of results reported in the last decade are shown). Almost all compounds were ineffective *in vivo* experiment even when they were very promising *in vitro.* This was also the case with α–phenylmyristic and oleic acids. The former showed no suppressing effect on tuberculous lesion in mice, and the latter rather some enhancing effect. Observations were also made on the ineffectiveness of fatty acids in the Kircher's medium containing serum albumin and some enhancing effect on the bacterial respiration with oleic acid-albumin complex.

These observations suggest that albumin may change the physicochemical properties of fatty acids which is unfavorable to the suppression of the growth of mycobacteria. But as the precise discussion on this problem is outside the scope of this paper, the author would like to describe it elsewhere.

Conclusion

All the 64 fatty acids and their derivatives including branched-chain fatty acids and halogen or rhodan substituted fatty acids were tested in vitro against *Mycobacterium tuberculosis var. Gallinaceum.* The most effective one was α–ethylmyristic acid. Their mode of action was discussed and the assumption was made that fatty acids have some physico-chemical properties responsable for the suppression of the growth of bacilli in the absence of albumin.

The author is deeplv indebted to Prof. K. Kodama for his continued active help in carrying out these series of work and also desires to thank Miss R. Kitamura for technical assistance in this investigation.

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ON THE DIGESTIBILITY OR RESORPTION-COEFFICIENTS OF VARIOUS FATS IN FOODS.

By

RYUZO IWATSURU and TARO NAKAMURA.

(From the Medical Clinic of Wakayama Medical College Wakayama, and the Laboratorium of Osaka National Sanatorium, Osaka)

(Received for publication, July 12, 1950)

In Osaka sanatorium were given the diets containing a large amount of various fats to the patients habitually parallel 3 in number, who were suffering from pulmonary tuberculosis in lungs of bronchogenous progressing mixed type and had no fever and were kept rest. Then the digestibility or the resorption coefficients of fats were experimentally calculated with the following results. Fats in diets were butter, cod-liver-oil, soy bean oil, palm oil, butter with soy-bean-oil, bonito with cod-liver-oil and pork. They were gradually added to a certain fundamental diet containing 1800 Cal. i.e., 80 g. in protein, 300–400 g. in carbohydrate and 20 g. in fat, from O g. at the beginning to 100∼150 g. at the end. The increase of additional fats was held at the rate of about 3 to 10 g. on steps every week. The daily total income was constant during the same week, for the patients were all kept in the week period with the same monotonous food.

On the other hand, we have determined the quanitites of fat in food as well as feces, with the Soxhlet's ether extraction method after dryness of the material. The average daily values of the feces fat in a week period are mentioned. The results will be found in the following table.

The diet of the first week of the experiments denotes respectively fundamental value, to which no supplementally fat was added. In the second week 10–20 g. of fat (Table I.) were added daily to

TABLE I. Digestibility or resorption coefficient of various fats in diets.

	Week Period.	1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.
Butter diet	F. D	17.84	38.22	58.52	78.09	96.82	114.74	133.01		
	R. C	76.36%	94.34%	95.97%	96.23%	97.31%	97.75%	96.77%		
Cod liver oil diet	F. D	15.99	23.48	32.91	40.09	48.13	54.26	64.22	82.37	104.73
	R. C					95.41	94.09	95.48	93.50	94.93
Soy bean oil diet	F. D	19.58	30.03	40.98	52.23	57.40	65.95	87.69	103.97	
	R. C	80.09	83.84	90.09	88.75	88.13	90.82	90.13	95.36	
Palm oil diet	F. D	16.88	29.68	39.61	45.24	67.68	102.50			
	R. C	74.26	82.34	83.54	85.06	88.13	94.11			
Butter and Soy bean oil diet	F, D	18.39	35.67	53.10	71.20	90.29	108.55			
	R. C	72.32	86.53	90.76	92 89	93.16	93.17			
Bonito and Cod liver oil diet	F. D	13.25	18.30	28.23	46.19	57.17	75.38			
	R. C	77.75	83.55	83.48	91.23	92.76	95.54			
Pork diet	F. D	9.02	49.10	60.19	135.64	138.43	168.72			
	R. C	75.17	93.40	94.39	96.82	96.83	96.79			

F. D. Fat in Diet. R. C. Resorption Coefficient.

TABLE II. Fats in feces and in diet.

	Week Period.	1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.
Butter diet	F. D	17.84	38.22	58.52	78.09	96.82	114.74	133.01		
	F. F	4.22	2.16	2.36	2.94	2.59	2.58	4.00		
Cod-liver oil diet	F. D	15.99	23.48	32.91	40.09	48.13	54.26	64.22	82.37	104.73
	F. F		2.07			3.17	3.12	2.26	5.14	5.30
Soy-bean oil diet	F. D	19.58	30.03	40.98	52.23	57.40	65.95	87.09	103.97	
	F. F	3.89	5.18	3.77	6.05	7.47	6.05	4.02	4.81	
Palm oil diet	F. D	16.88	29.68	39.61	45.24	67.68	102.50			
	F. F	4.11	4.85	6.55	6.72	8.20	6.03			
Butter and Soy bean oil diet	F. D	18.39	35.67	53.10	71.20	90.29	108.55			
	F. F	5.09	4.60	4.91	5.10	6.17	7.41			
Bonito and Cod liver oil diet	F. D	13.25	18.30	28.23	46.19	57.37	75.38			
	F. F	2.91	3.01	3.28	4.05	4.10	3.36			
Pork diet	F. D	9.02	49.10	69.19	135.64	138.43	168.72			
	F. F	2.24	3.24	3.19	4.33	4.78	5.41			

F. D. Fat. in diet. F. F. Fat in feces.

the fundamental diet, and the third week 20–40 g. and so on. From our experiment, we can understand the following result. In such cases the richer the added fat are in diet, the better they are absorbed from the intestine. The coefficient of resorption or digestibility do not fall according to the increase of ingesting fats in diet. At last, they have reached the high coefficient of 93–96% for either animal fats or vegetable oils. With regard to the coefficients it was believed for a long time, that the vegetable oils (soy-bean-oil and palm oil) are taken for worse than the animal fats (butter and cod-liver-oil), but from our experiments we can understand that they are very good for the absorption of intestine.

Subsequently, why does the coefficient of resorption ascend according to the increase of ingesting fats in diets? In the other hand, it must be accepted that the more the coefficient ascends, the excretion of fats in feces decreases. In our experiments the excreted fat in feces was actually measured and then the coefficient of resorption was calculated, when certain diets with various fat content were given, with following results.

It is obvious from these figures that the excreted fat amount in feces is the same and generally fixed in each other. The mean value of fats in feces was calculated, as appears below.

TABLE III. Mean value of excreted fats in feces. C g.

	C		C
Butter	2.98	Butter and Soybean oil	5.58
Codliveroil	3.51	Bonito and Codliver oil	3.46
Soybeanoil	5.15	Pork	3.79
Palm oil	6.08		

Meanvalue of these figures C = 4.36

These figures show, in fact, that the digestibility of animal fats is better than that of vegetable oils. Moreover, from these results, it is obvious that the increase of coefficient of resorption may be due to the constant excretion of fats in feces. Since the fat amount in feces per day are constant, the greater the fats in diets, bring about the smaller coefficient of excretion. Naturally, the digestibility or the coefficient of resorption of foods may be different by the influence of many conditions in the environment, internal and external, in which man is living. The common idea would seem to justify the popular supposition that normal feces are made up of the undigested residues from the foodstuffs. However, it is far from fact. The feces are, after Sherman, chiefly the unabsorbed residues of intestinal excretions.

When the definite excretion of fats in feces be c and the deviation Δc, the total excretion will be given by the $c+\Delta c$.

If the coefficient of excretion of fats in feces is y and the fat amount in diets is x, we have the following formular.

$$xy=c+\Delta c$$

But, it has been elucidated that Δc is very small in our experiment. The increase of fats in diets was held in the same way as the above described. And on reaching 100 g. of fats in diets, it was gradually and conversely decreased to 30 g. of fats. The results are as follows.

It is seen from this experiment that fecal fats tend to diminish to the definite point by the different conditions, even though the fats are added in great quantitiy. It is supposed that Δc in $c+\Delta c$ becomes very small step by step and the fecal fat is consequently represented by c, when all conditions, physical and mental, internal and external, are perfectly adapted to the absorption of fats in the intestine. Hence, $xy=c+\Delta c$ becomes $xy=c$ in the perfect conditions. C is the constant value for an individual and the fats in feces.

If c may be represented with the mean of excreted fats in feces,

TABLE IV. Excretionscurve of fat in feces when fats in diets increased and decreased.

Week Period.	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Fats in diet	16.88	29.68	39.61	45.24	67.68	102.80	67.68	45.24	39.61
Fat in Feces	4.11	4.85	6.55	6.92	8.33	6.03	5.17	4.89	4.56
Excretion Coefficient	25.67%	17.66%	16.46%	14.94%	11.87%	5.89%	7.65%	10.81%	11.51%

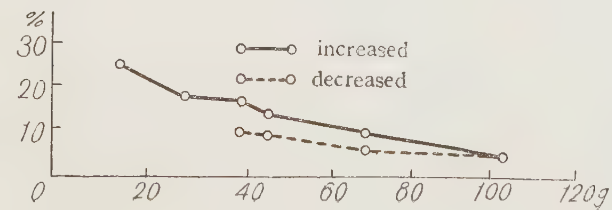


TABLE V. Calculated excretion coefficients at the time of various fats in diets.

	Week Period.	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Butter diet	F. D	17.84	38.22	58.52	78.09	90.82	104.74	133.01		
	C. E. C	16.70%	7.79%	5.09%	3.82%	3.08%	2.59%	1.95%		
Cod liver oil diet	F. D.	15 99	23.48	32.91	40.09	48.13	54.26	64.22	82.37	104.73
	C. E C.	21.95%	14.94%	10.66%	8.75%	7.29%	6.46%	5.46%	4.26%	3.35%
Soy bean oil diet	F. D	19.85	30.03	40.98	52.23	57.40	65.95	87.69	103.97	
	C. E. C	26.30%	17.14	12.56%	9.86	8.97%	7.81%	5.87%	4.95%	
Palm oil diet	F. D	16.88	29.68	39.61	45.24	67.68	102.50			
	C. E. C	36.18%	20.48%	18 43%	13.43%	8.98%	5.93			
Butter and Soy bean diet	F. D	18.39	35.67	53.10	71.20	90.29	108.55			
	C. E. C	30.43%	15.64%	10.51%	7.78%	6.18%	5.14%			
Bonito and Cod liver oil diet	F. D	13.25	18.30	28.23	46.19	57.17	75.38			
	C. E. C	26.11%	18.90%	12.26%	7.49%	6.05%	4.57%			
Pork diet	F. D	9.02	49.10	68.19	135.64	138.43	168.72			
	C. E. C	40.02%	7.71%	5.47%	2.79%	2.73%	2.24%			

F. D. Fat in diet C. E. C. Calculated excretion coefficient.

the calculated coefficient of excretions by the $xy=c$ are as follows.

In the case of the palm oil diet, diet in butter and soy bean oil and pork diet the calculated coefficients are higher than the experimental coefficients. But this may be caused by the different kinds of fats, from the fats contained in the fundamental diet. Many curves of the coefficient of excretion show the relation of each other between the added fats and oils in diets. On the other hand the coefficients of excretion are completely agreeable experimentally and calcurably. The following curves show these relations.

TABLE VI. Butter.

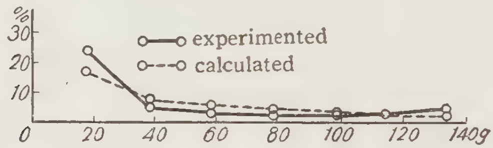


TABLE VII. Cod liver oil.

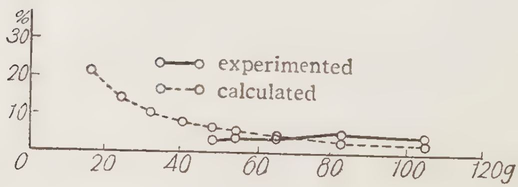


TABLE VIII. Soy bean oil.

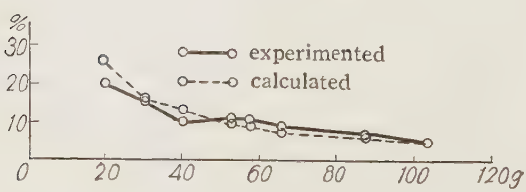


TABLE IX. Palmoil.

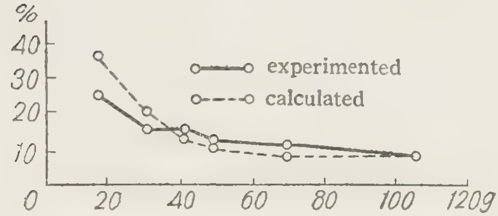


TABLE X. Butter and soy bean oil.

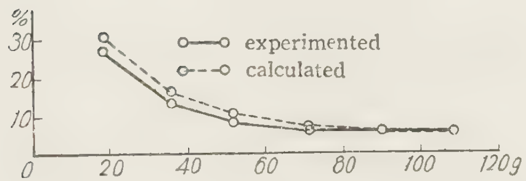


TABLE XI. Bonito and cod liver oil.

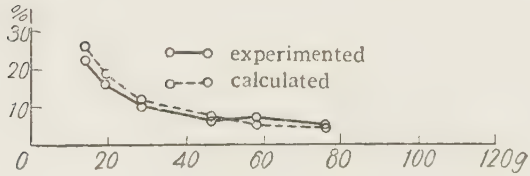
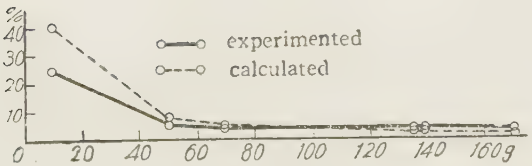
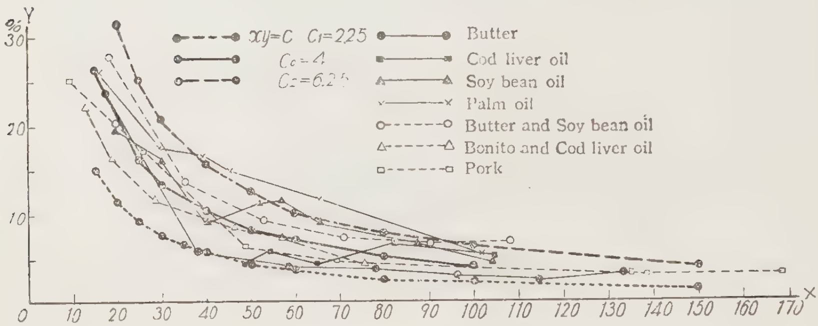


TABLE XII. Pork.



Even if the constant value c changes more or less in every person in spite of the quantity and the quality of added fat in diet, seeing that the curves obtained experimentally are inserted by two curves of $xy=c$ ($c=2.25$ and $c=6.25$). The significance of 2.25 and 6.25 will be explained in the following. We shall draw two curves.

TABLE XIII. Curve of coefficient of fat excretion.



When a line which divides equally the angle XOY, is drawn in the upper table, this line will cross at the point of c_1 and c_2, with the two dotted curves. Then oc_1 is 2.25 and oc_2 is 6.25. All curves of coefficient of fat excretion would be inserted in the two curves ($xy=2.25$ and $xy=6.25$).

It is no doubt that c exists within the limits from 2.25 to 6.25 and, if c will change from 2.25 to 6.25 at an interval of 0.25 and if x will change from 15 to 100 at the same time, y may be represented in the upper tables.

If these figures in the table are compared with the coefficient in the upper experiments, c will be perhaps within the limits from 2.5 to 6.0, c_0 is the middle point and may be observed as the mean value of the excreted fats in feces. It is fact that c_0 is perfectly agreeable with oc_0 in the curve of $xy=4$. In this experiment $c=4.36$ was acquired as the mean value. If $c=4$ will be assumed as the

coefficient of excretion of various fats in diets, the digestibility and the fats absorbed from the intestine will be briefly determined for the practical purposes. Also, if we shall determine the fats excreted in feces with the diets containing various fats ....butter, cod liver oil, soy bean oil and palm oil ...., the mean of digestibility will be given each other.

SUMMARY

Our results would be concluded in the followings.

1. The excreted fats is constant, even if various sorts of fats are added to the fundamental diets (to 150 g.)
2. The excreted fats in feces............................ c g.
   Ingesting fats in diets............................ x g.
   a) Coefficient of excreted fats in feces............ y %

   $$y=\frac{c}{x}\times 100$$

   b) Digestibility of fats in diets............ y %

   $$y=(1-\frac{c}{x})\times 100$$

   c) Absorbed fat............ y g.

   $$y=x-c$$

SUPPLEMENT

1. An experiment of fat-free diet is now being held in our sanatorium and will be soon reported.
2 Papers on carbohydrate and protein diet would be nearly published.

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ON THE DIGESTIBILITY OF PROTEINS IN DIETS.

By

RYUZO IWATSURU, TARO NAKAMURA and TATSUO INOKUCHI.

(From the Medical Clinic of Wakayama Medical College, Wakayama, and the Laboratorium of Osaka National Sanatorium, Osaka)

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In the article concerning the digestibility of fats in diet we have already shown that various fats are absorbed in the intestinal canal up to 98–100 per cent and that these percentages represent net coefficients of resorption while the coefficient as generally assumed remains to be of gross coefficient. Moreover we have affirmed that the excreted fats in feces are constant and that, if ingesting fats in diet are x g. and excreted fats in feces c g. the coefficient of digestibility of fat in diet, y is $1-\frac{c}{x}$. In this article we will report the coefficient of resorption of proteins in diets.

Digestibility of proteins

Iwatsuru reported the above described experiments as a special lecture for the congress of the "Japanese Tuberculosis Association" in April 1948 in Tokyo. Here we shall illustrate three following experiments regarding the protein digestibility. The first case is an experiment of diet containing bonito and cod liver oil. The second case is an experiments of diet containing pork and the third case is an experiment of diet containing a great quantity of protein. Various sorts of protein were added to the fundamental diet. At the first and the second case a monotonous foodstuff......... bonito and pork was added. But at the third case a certain mixture of proteins...... ..animal and vegetable proteins.........was added.

Foods of animal proteins were namely salted salmon and dried sardine and the vegetable one soy bean, miso and bean curd. (The ratio of animal protein to vegetable one was 1:1.) In all our cases the ingesting fundamental diet contained a definite amout of carbohydrate, while in the first case the mixing ratio of protein to fat, which were contained in diet, was 1:1 and in the second case the ratio 2:1. In the third case fat was 30 g. and proteins were gradually added to the fundamental diet at the ratio of 25 g. every week. Ingesting proteins in the first case and the second case were increased at the rate of 10 g. The increase of ingesting protein has reached 170 g. (One person hated a diet containing a great deal of protein over 170 gr. and could not eat this cost entirely.) Subsequently we collected and dried the ingesting diets or excreted feces every day or every week and determined the amount of nitrogen by Kjeldahl method, which gives us naturally the value of protein in diet by multiplying with 6.25. We have selected three tuberculous patients without fever and have administered these diets to them. The figures of the tables I, II, and III demonstrate the digestibility of proteins.

In the experiments of a diet rich in various fats, the digestibility of fat became 93–98 per cent, while that of proteins have been 85–87 per cent and not increased more than it. These results of our experiments were obtained from 50 to 100 g. proteins. Then, the more the ingesting protein increases, the greater the digestibility of protein happens. No alteration was found in the third case. It is necessary to know that in the first and the second case ingesting protein was 50–90 g. while in the third case it was 90–170 g. Therefore we can conclude that the increase of ingesting protein improves the digestibility in some part and in another part it does not become better. On the curious point we shall describe in the following chapter.

TABLE I. 1st Experiment

Week Period		1st	2nd	3rd	4th	5th	6th
P. D.		53.06	62.50	70.06	80.81	87.68	88.88
R. C.	Hi	77.84	81.98	86.79	79.69	86.83	81.53
	Su	77.24	77.71	81.24	86.01	82.32	87.37
	Yo	77.76	81.68	85.52	88.95	81.84	87.53

P. D. Protein in diet R. C. Resorption coefficient

Hi, Su, Yo are the names of tuberculous patients

TABLE II. 2nd Experiment

Week Period		1st	2nd	3rd	4th	5th	6th
P. D.		54.72	58.15	64.62	72.99	80.43	87.18
R. C.	Ko	77.61	78.51	79.99	78.43	81.66	84.74
	Ta	78.88	79.06	80.66	83.56	83.84	88.53
	Mi	80.36	79.06	79.21	80.14	81.52	84.31

P. D. Protein in diet R. C. Resorption coefficient

TABLE III. 3rd Experiment

Week Period		1st	2nd	3rd	4th	5th
P. D.		72.37	92.81	113.31	136.43	171.56
R. C.	No	87.14	85.06	84.73	84.65	84.05
	Yo	88.78	85.39	85.61	85.53	83.54
	Ni	82.59	83.57		86.30	85.87

P. D. Protein in diet R. C. Resorption coefficient

2) Excreted proteins.

In general, it is obvious that the amount of ingesting and excreted proteins would determine the coefficient. In our experiments we have determined the amount of ingesting protein and of the excreted protein in feces to understand the digestibility. (Consult the following Tables).

Table IV. 1st Experiment

Week Period		1st	2nd	3rd	4th	5th	6th
P. D.		53.06	62.50	70.06	80.81	87.68	88.88
P. F.	Hi	10.68	11 26	9.19	16.41	11.72	11.97
	Su	12.08	13.87	13.29	11.31	15.51	11.31
	Yo	11.80	11.45	10.15	9.93	16.10	11.08

P. D. Protein in diet P. F. Protein in feces

Table V. 2nd Experiment

Week Period		1st	2nd	3rd	4th	5th	6th
P. D.		54.72	58.15	64.62	72.99	80.43	87.18
P. F.	Ko	12 25	12.50	12 93	15.75	14.75	13.68
	Ta	11.56	12.18	12.50	12.00	13.00	10.00
	Mi	10.75	12.18	13.43	12.31	14.87	13.68

P. D. Protein in diet P. F. Protein in feces

TABLE VI. 3rd Experiment

Week Period		1st	2nd	3rd	4th	5th
P. D.		72.37	92.81	113.31	136.43	171.56
P. F.	No	9.31	13.87	17.31	21.62	27.37
	Yo	8.12	13.56	16.31	19.75	28.25
	Ni	12.06	15.25	18.93	18.56	24.25

P. D. Protein in diet P. F. Protein in feces

From our results the excreted protein in the first and the second case was equal but in the third case gradually increased. It seems that the excreted protein in feces is constant from 50 to 90 g. of ingesting proteins without relation with the increase of it, while on the case of over 90 g. of ingesting proteins the excreted protein in feces gradually increases and therefore the coefficient has not altered. If so, 1) from 50-90 g. the amount of ingesting proteins determines the digestibility, 2) over 90 g. it is independent on the ingesting proteins.

It was commonly supposed that the normal feces are made up of the undigested residues of the foodstuffs and of the digestive juices, intestinal epithel and bacterias. However this is very far from the fact. After Sherman the feces are chiefly the unabsorbed residues of intestinal excretions. Of course, perfect unabsorbed residues of foodstuffs exist in feces, but it may be probably slight. From this point of view the net coefficient of resorption be 98–100 per cent and ingesting proteins almost perfectly absorbed from the intestine. On the other hand it is also probable that the digestibility may be caused by a compensating state of the digestive tract. For we observed a steady increase of nitrogen in feces, as soon as the ingesting proteins exceed over 90 g. We suppose that the increase of fecal nitrogen will be due to the urea, ammonia and uric acid,

instead of protein, when the ingesting proteins are beyond 90 g. (We will perform the analysis of nitrogenous substances in feces in the subsequent paper.)

As has been previously mentioned, feces contain digestive juice, mucus, intestinal epithel and bacteria in the digestive tract and the nitrogen excretion would be influenced by these factors. But if we could conclude that the amount of excreted protein in feces is constant, we may distinguish the concept between the net and gross coefficient and illustrate the meaning of the digestibility like fat.

Relation between ingesting and excreted proteins (mainly nitrogen) in adequate quantity

As the digestibility of fat has a certain relation with ingesting and excreted fat and the excreted fat is constant, so the digestibility increases corresponding to the increase of ingesting fat. When we draw a graph of the digestibility, it will become hyperbola. As mentioned in thei previous chapter, the amount of excreted proteins in feces is constant, as the fecal nitrogen is about 2 g. daily, so we can naturally suppose that the graph of coefficient of proteins is hyperbola like fat. In the experiment of the diet containing pork we analysed the proteins in feces every day. The result of excretions described in table VII. denotes the mean daily value for a week. The following table which was obtained with the diet containing pork, illustrates that the fecal nitrogen is 2 g. daily.

Supplement

We have measured the fecal N in many cases, the results of which are pointed out as follows in order to understand that the fecal N is constant in diets containing various proteins.

TABLE VII. The amount of ingesting proteins and the amount of N in feces (Pork-diet)

		N	T	M
Date	N in Diet	N in feces.	N in feces.	N in feces.
1 14	8.72	2.54	2.89	2.73
15		1.66	1.82	1.07
16	(Protein	1.73	1.39	2.13
17	54.72)	2.05	1.12	1.66
18		2.44	2.22	1.35
19		2.54	1.88	1.44
20		1.46	2.02	1.25
21		1.28	1.45	2.10
22	9.31	3.31	2.52	2.19
23		2.19	2.18	1.49
24	(Protein	2.30	1.59	1.74
25	58.15)	1.00	1.25	2.14
26		1.01	2.44	1.94
27		2.45	2.24	1.75
28		1.80	1.48	2.38
29	10.25	2.53	1.55	1.75
30		2.32	2.10	2 25
31	(Protein	2.47	2.28	2.41
2 1	64.62)	0.64	2.05	2.32
2		2.41	2.09	2.02
3		1.98		
4	11.68		2.42	1.65
5		2.55	2.33	1.53
6	(Protein	2.59	2.57	2.42
7	72.99)	2.91	2.93	2.40
8		3.42	2.53	1.78
9		3 82	1.39	2.19
10		1.38	2.27	1.82
11	12.87	2.05	1.96	2.39
12		2.61	1.23	2.27
13	(Protein	2.06	1.76	2.15
14	80.43)	3.36	3.25	3.06
15		2.17		2.65
16		2.26	2.35	1.98
17		2.02	1.92	2.19
18	13.95	2.04	2.28	2.53
19		1.42	1.62	2.28
20	(Protein	2.00	1.33	1.87
21	87.18)	2.75	0.75	1.42
22		2.11	1.91	2.08
23		2.83		2.15
24		1.81	1.72	3.06

TABLE VIII. Protein in feces when used in mixed diet.
(Protein 40—90 g.)

Protein in Diet (g.)	40	50	60	70	80
	8.58	11.52	12.95	10.80	14.20
Protein in feces (g.)	11.17	12.28	13.58	9.39	12.45
		9.11	13.11	10.98	12.55
(N×6.25)		11.52	13.12	11.90	14.44
		8.10	8.98	10.69	11.45
		10.31	12.19	11.75	
		12.97	9.37	14.74	
		12.71	9.62	13.35	
		12.96	10.66	12.28	
		10.72	10.90	15.03	
		10.25	7.73	11.83	

DISCUSSION

Hitherto the digestibility was illustrated as follows. As unabsorbed food residue is excreted as feces, so the coefficient of excretion of food can be calculated as the ratio of unabsorbed food residue to ingesting food. If so, the digestibility is acquired by reducing the coefficient of excretion from 100%. These coefficients have determined the nutritive value of food, as following Atwater's result point out.

Generally speaking, animal proteins are better than vegetable proteins about the absorption in intestines and then it is supposed that they have the more nutritive value. Sherman indicated that in some cases these figures of vegetable proteins are higher than have been reported for similar food by other observers and that the coefficients differ less for the different types of food than might be expected from popular impression of digestibility. Moreover it is probable that the digestibility are less influenced by the conditions, under which the food is eaten and vary less with individuals than is genarally supposed. Then we affirm that the feces are chiefly the unabsorbed residues of intestinal excretions. Thomas reported that

the fecal N was not the food residue and that the absorption of animal proteins, is perfect and vegetable proteins, too, when broken up into pieces. We do not know regarding the chemistry of these nitrogenous compounds excreted into the intestine. Then we conclude that the feces are derived principally from the substance excreted through the wall of intestine with the results above described.

In any kinds of foods, fecal N is constant and the amount of ingesting proteins does not influence upon the absorption from the intestine. Then we suppose that the absorption percentage of proteins be 98–100% like fat. It is due to the constancy of N excretion that the more the ingesting amount of proteins increases, the greater the gross coefficient is. It is clear that a diet does not probably consist of a protein only but contains various proteins in many cases and that the difference of the nutritive value between animal- and vegetable proteins is not according to the fecal N. Various values of fecal N were given by many other observers. Inoue reports that the fecal proteins are 13.60, 15.30 and 14.35. (cf., 2.18, 2.79 and 2.45 as N), when proteins 58.52 gr. are eaten. In this case

TABLE IX. Average coefficients of resorption of foods when used in mixed diet.

Animal foods	97 %
Cereals and breadstuffs	85
Dried legumes	78
Vegetables	83
Fruits	85
Total food of average mixed diet	92

the foodstuff consists of carbohydrate 341.6 g. and fat 11.94 g. From Takahashi the fecal excretion is 6.1 g. of nitrogen per day, when a person is taking a diet containing 183 g. of carbohydrate, 140 g. of fat and 316 g. protein (50.6 g. of N) as soy bean. We are able to understand that these results correspond to the gradual straight

increase of fecal N in our experiment. (Of course we don't know about the constituents of nitrogenous substance and suppose that ammonia, urea and uric acid in the feces as described in the chapter of digestibility of proteins). When we compare the above described results of our experiments with these figures, the case of Inoue corresponds to the first and the second case and the Takahashi's case to our third case. From our results and the other by many observers the absorption of proteins may increase gradually outwards, when a diet contains various proteins to 85–90 g. The net coefficient of resorption of proteins be 98–100%. It seems that the fecal N is not constant concerning the fecal N excretion, when a diet contains over 85–90 g. of proteins. Sherman reports that the fecal N is 0.5–0.6 g. per day, that it is more than by starvation and richer than with a diet of fat and carbohydrate alone frequently. Wallace and Salomon have administered 250 g. of cane sugar daily to normal persons and have obtained the amount of fecal N 0.53–0.38 g. Von Noorden's patient, suffering from tuberculosis, without fever, when taking 11.6 g. of N daily showed the fecal N 0.92 g. Austin, Ordway and Montague studied the utilization of food by three consumptives with the proportion of proteins and fats varied. The excretion N was obtained with following results.

TABLE X. Absorptive Power of Digestive Tract in Three cases of Phthisis.

Period	Nitrogen intake	Nitrogen in feces	Nitrogen lost in feces (%)
		case I	
1	33.22	0.39	1.18
2	31.86	0.36	1.13
3	31.64	0.39	1.25
		case II	
1	33.08	0.49	1.49
2	35.50	0.54	1.53
3	29.91	1.26	4.21

case III

1	29.71	0.64	2.17
2	28.963	1.03	3.51
3	25.52	0.91	3.56

It shows that the excretion of nitrogen in feces does not increase when a diet considerably rich in protein is taken. In this experiment the digestibility is more than 90%. Then we suppose that the base of excreted N in feces in our country may be greater than in foreign. The question will be understood how and when the nitrogenous compounds may be excreted into intestine. The fecal N alters owing to the diet containing various proteins, so it may be probable that our results are different from the many results of the foreign literature. The ingesting proteins in the intestine be almost perfectly absorbed and its net digestibility be 98–100%. The fecal N is about 2 g. (12.5 g. as protein) in any kind of diet containing various proteins.

Summary

1) The excretion of fecal N as protein constituent is constant, independent on the amount of the ingesting proteins.

2) The ingesting proteins may be absorbed in the intestine for 98–100 per cent.

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The Journal of Biochemistry, Vol. 37, No. 4.

THE DETERMINATION OF α-KETOGLUTARIC ACID IN BLOOD AND URINE

By

TAIJI SHIMIZU

(From the Department of Biochemistry, School of Medicine, Niigata University, Niigata)

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As α-ketoglutaric acid is an important intermediate metabolite of carbohydrate and amino acids, there is a peculiar biochemical interest in the determination of the concentration of this acid in blood and urine. Heretofore, the gravimetric determination of this acid was described by Krebs (1933) (1), Weil-Malherbe and Krebs (1935) (2), Simola (1936) (3), and, Martius and Knoop (1937) (4), using the property of this acid to form the hydrazone with 2, 4-dinitrophenylhydrazine, which precipitates in acid solution. Besides, Krebs (1938) (5) oxidized the 2, 4-dinitrophenylhydrazone to succinic acid and determined manometrically the carbon dioxide produced from it by the action of succinic dehydrogenase. However, these methods are not satisfactorily applicable to our purpose on the points of specificity and sensibility. Recently, Friedemann and Haugen (1943) (6) reported the new method to determine α-ketonic acids in blood and urine. This method was based on the difference of the rate of reaction of α-ketonic acids with 2, 4-dinitrophenylhydrazine and the difference in solubility of the 2, 4-dinitrophenylhydrazones of α-ketonic acids in various solvents. Final readings were made by photoelectric colorimeter after the addition of alkali to the extract containing the hydrazone. The concentration of α-ketoglutaric acid was calculated from the values of pyruvic acid and the total ketonic acids measured colorimetrically, using several kinds of light filters. Refering to this report, I have devised a new method of the

determination of α-ketoglutaric acid. Pyruvic acid reacts with 2, 4-dinitrophenylhydrazine completely within 5 minutes at 25° but α-ketoglutaric acid needs 25 minutes at 25° to complete the reaction. 2, 4-Dinitrophenylhydrazone of pyruvic acid is well soluble in xylene, but that of α-ketoglutaric acid is hardly soluble in xylene. Making the best use of these empirical facts, the new method was divised as follows. The 2, 4-dinitrophenylhydrazone of pyruvic acid, which was formed with 2, 4-dinitrophenylhydrazine within 5 minutes at 25° was extracted by means of xylene, and some 2, 4-dinitrophenylhydrazine was added further to the remaining solution, and the mixture was kept at 25° for 25 minutes. The 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid thus produced was extracted by means of ethyl acetate, and the extract was made strongly alkaline with the addition of sodium hydroxide. The developed brown red color was measured colorimetrically. The acetoacetic acid, whose 2, 4-dinitrophenylhydrazone was also produced within 5 minutes at 25° but which was hardly soluble in xylene, was decomposed with aminoantipyrine, as described by Kaneko (1938) (7), thus avoiding any interference from β-ketonic acids in the final reading.

Method

(*1*) *Reagents.*

10% Trichloroacetic acid solution: stored in a refrigerator. Trichloroacetic acid must be purified by distillation.

Xylene: washed with concentrated H_2SO_4, and redistilled after the addition of solid alkali.

Ethyl acetate: redistilled, after removals of acid by shaking with concentrated NaOH, and, of alcohol, by shaking with 50% $CaCl_2$ solution.

2, 4-Dinitrophenylhydrazine reagent (DNP-reagent): 0.5 g. of 2, 4-dinitrophenylhydrazine dissolved in 100 ml. of 2 N-HCl by heating with reflux condenser, shaking gently, and filtered after cooling.

Stored in a refrigerator.

0.002% Aminoantipyrine solution: an aqueous solution of aminoantipyrine in 0.002% concentration, prepared with aminoantipyrine hydrochloride.

10% Sodium carbonate solution: prepared with anhydrous sodium carbonate.

Saturated sodium hydroxide solution

20 N-sulfuric acid

Acid clay (Takeda): used after previously being dried at 140°.

To obtain the extinction-concentration curve, the standard solution of α-ketoglutaric acid in 0.1 N sulfuric acid is used. It must be stored in a refrigerator. The α-ketoglutaric acid used is synthesized by the decomposition of oxalosuccinic ester with fuming hydrochloric acid, following the method of Neuberg and Ringer (8).

(2) *Procedure.*

Blood is withdrawn from the vein by means of 2 ml. slender syringe which is calibrated previously. 2 ml. of the sample are rapidly ejected in a fine stream through the needle into 10 ml. of 10% trichloroacetic acid solution and is deproteinized by centrifugation. 8 ml. of the clear supernatent solution are transferred to a 25 ml. centrifug tube. The temperature of the content is adjusted to 25° in a water bath, and 0.7 ml. of DNP-reagent is then added, and the mixture is allowed to react for 5 minutes at 25° 8 ml. of xylene are now added to it, and the mixture is stirred strongly by a rapid stream of air through a capillary tube for 3 minutes. After being separated into two phases by the centrifuge, the lower aqueous layer is transferrd to an another centrifuge tube by means of a capillary pipette. The upper layer is used to determine the pyruvic acid (6) (9).

To the aqueous layer 1 ml. of 0.002% aminoantipyrine solution is added and the mixture is allowed to stand for 15 minutes at 37° After the temperature of the mixture is adjusted to 25° in a water bath, 0.2 ml. of DNP-reagent is added to it, and the mixture

is allowed to react for 25 minutes at 25° 3 ml. of xylene are now added and a rapid stream of air is passed through the mixture for one minute. The lower aqueous solution is then transferred to an another centrifuge tube by a capillary pipette, 10 ml. of ethyl acetate are added to it and a rapid stream of air is again passed through the mixture. After being separated into two phases by the centrifuge, most of the lower aqueous layer is removed by means of a capillary pipette, and the ethyl acetate layer is washed with 3 ml. of water. After removing the water, 3 ml. of water is again added, and the mixture is placed in a refrigerator for 18 to 24 hours. The aqueous layer is removed and exactly 6 ml. of 10% sodium carbonate solution are added to the ethyl acetate layer. After mixing, the two phases are allowed to separate. A 5 ml. pipette is inserted quickly through the upper layer; air is momentarily blown through the pipette to discharge the small quantity of the ethyl acetate in it. The aqueous phase is then drawn into the pipette, and transferred to a test tube. Exactly 2 ml. of saturated sodium hydroxide solution are added and the contents of the tube are immediately mixed. Readings are taken by means of Pulfrich's stufenphotometer with the light filter S 47, at the lapse of 5 to 15 minutes after the saturated NaOH was added.

In determining the amount of urinary α-ketoglutaric acid, 0.5 ml. of 20 N H_2SO_4 is added to each 100 ml. of urine, and to 10 ml. of the acidified urine 0.75 g. of acid clay is added. The mixture is shaken violently for 2 minutes and then filtered. The filtrate is mixed with 5 times its volume of 10% trichloroacetic acid solution and 8 ml. of the sample prepared in this way are used in the determination.

The α-ketoglutaric acid content of urine is not changed appreciably during 24 hours of storage at room temperature, and the sample treated with trichloroacetic acid will remain unchanged with respect to α-ketoglutaric acid for at least another 24 hours when kept cold.

Experimental

Extraction Degree—To determine whether the extractions of the hydrazone of α-ketoglutaric acid with ethyl acetate and sodium carbonate are complete or not, the following experiment was undertaken. The extinction coefficient E_0 was measured, carrying out the determniation without addition of DNP-reagent and using water instead of blood. Then the determination was made with the 7.12 mg./dl standard solution of α-ketoglutaric acid following the above method, and after the extraction of hydrazone with ethyl acetate, the second extraction was carried out with ethyl acetate, and the extinction coefficint E_A was measured with this second extract. Furthermore, with the same standard solution, after the extraction of hydrazone from the ethyl acetate layer with sodium carbonate, the second extraction was carried out by means of sodium carbonate, and the extinction coefficient E_N was measured with this second extract. As shown in Table I, the extractions by both 10 ml. of ethyl acetate and 6 ml. of 10% sodium carbonate were complete.

Table I

Filter	E_A-E_0	E_N-E_0
S 47	−0.001	−0.002
S 50	−0.001	−0.001
S 53	+0.002	+0.001

The Relation Between Concentration and Extinction Coefficient—Using the standard solution of the concentrations of 1.78 mg./dl, 3.56 mg./dl, 5.34 mg./dl and 7.12 mg./dl, extinction coefficients were measured with light filters S 47, S 50, and S 53. The results are shown in

Table II. The relation between the extinction coefficients and the concentrations was linear and the ratio of extinction coefficient/concentration was maximum with the filter S 47.

TABLE II

Extinction coefficient with Filter	Concentration of α-ketoglutaric acid (mg./dl)					Extinction coefficient / Concentration
	0	1.78	3.56	5.34	7.12	
S 47	0	0.085	0.162	0.245	0.326	0.0463
S 50	0	0.060	0.126	0.183	0.243	0.0345
S 53	0	0.056	0.117	0.173	0.237	0.0325

The Degree of Color Developments of Various Compounds—Acetone (100 mg./dl), oxaloacetic acid (6.3 mg./dl), β-hydroxybutyric acid (7.9 mg./dl), lactic acid (100 mg./dl), glucose (100 mg./dl), fructose (100 mg./dl), ascorbic acid (4.03 mg./dl), methylglyoxal (5.7 mg./dl), phenylpyruvic acid (398 mg./dl) and p-hydroxyphenylpyruvic acid (14.8 mg./dl) had no effect on the results of this determination. The effects of pyruvic, acetoacetic and dehydroascorbic acids are shown in Table III. The concentrations found are expressed in terms of α-ketoglutaric acid. Thus the latter three acids had almost no influence on the value of α-ketoglutaric acid, unless their concentrations were remarkably high. To make the influence minimum, the filter S 47 was found to be the most suitable.

TABLE III

	Concentration (mg./dl)	Filter	Extinction coefficient	Concentration found* (mg./dl)
Pyruvic acid	9.4	S 47	0	0
		S 50	0.018	0.52
		S 53	0.004	0.12

α-Ketoglutaric acid	3.56	S 47	0.165	3.56
+		S 50	0.127	3.68
Pyruvic acid	4.7	S 53	0.116	3.57
		S 47	0.060	1.29
	118	S 50	0.060	1.74
		S 53	0.037	1.20
Acetoacetic acid	60	S 47	0.005	0.11
	5.9	S 47	0	0
		S 50	0	0
		S 53	0	0
α-Ketoglutaric acid	3.56	S 47	0.165	3.56
+		S 50	0.128	3.71
Acetoacetic acid	5.9	S 53	0.116	3.57
	30	S 47	0.004	0.09
Dehydroascorbic acid		S 47	0	0
	8.82	S 50	0	0
		S 53	0	0

* All figures are expressed as α-ketoglutaric acid.

The Effect of Acid Clay—0.75 mg. of Japanese acid clay (Takeda) which was previously dried at 140° was added to 10 ml. of the 7.12 mg./dl standard solution of α-ketoglutaric acid. The mixture was shaken for 2 minutes and filtered. The α-ketoglutaric acid in the filtrate was estimated in order to determine whether its concentration is diminished or not. Next, the values were compared between the urine samples treated and not treated with acid clay. The recovery test was also carried out with urine. The blank value was measured, adding acid clay to the urine without DNP-reagent. All these results are shown in Table IV. According to the results, the acid clay treatment had no influence to the content of α-keto-glutaric acid of the pure solution. In the case of urine, the acid clay treatment distinctly lowers the obtained value. But, as the recoveries were all above 98% also in these cases, the higher values

of the urine sample without the treatment with acid clay must have resulted from other substances than α-ketoglutaric acid and the values obtained from the sample treated with acid clay can be concluded to be true. The blank value of urine measured without DNP-reagent after the treatment with acid clay showed zero. These facts indicate that the substances which interfere with the determination and do not react with DNP-reagent are all removed by the addition of acid clay.

TABLE IV

		No acid clay	Treated with acid clay	
		found (mg./dl)	found (mg./dl)	recovery (%)
I	α-Ketoglutaric acid 7.12 mg./dl		7.12	
II	Blank	0	0	
III	Human urine (1 : 2)	1.36	1.14	
	do. + α-Ketoglutaric acid 3.56 mg./dl		4.64	98.3
IV	Human urine (1 : 2)	1.32	1.10	
	do. + α-Ketoglutaric acid 3.56 mg./dl		4.61	98.6
V	Rabbit urine (1 : 2)	3.67	2.50	
	do. + α-Ketoglutaric acid 3.56 mg./dl	7.07	5.99	97.9
	Same urine without DNP	0.63	0	

5) *The effect of aminoantipyrine:* The aqueous solution of acetoacetic acid was taken as the sample of the determination, and the results were observed under the following conditions. (i) Just as the procedure above mentioned, the aqueous solution was treated with aminoantipyrine at 37° for 15 minutes after the extraction with xylene, and the ethyl acetate extract was allowed to stand in a refrigerator for 18 to 24 hours. (ii) The treatment in a refrigerator was omitted. (iii) The treatment with aminoantipyrine was omitted. (iv) Both treatments were omitted. Table V indicates the results of the above four cases. From these results it can be concluded that the interference of acetoacetic acid is avoided almost completely by previously treating the sample with aminoantipyrine and keeping the ethyl acetate extract in a refrigerator for 18 to 24 hours. As for the effect of these treatments on α-ketoglutaric acid, experiments showed that they had only a very slight effect on the value of estimation for α-ketoglutaric acid (Table V).

TABLE V

Sample	Treatment with aminoantipyrine	Treatment with refrigerator	Found*
Acetoacetic acid			mg./dl
(i) 5.9 mg./dl	at 37° for 15 min.	for 18-24 hrs	0
(i) 60.0	do	do	0.11
(i) 118.0	do	do	1.29
(ii) 118.0	do	—	2.51
(iii) 60.0	—	for 18-24 hrs	5.00
(iv) 7.14	—	—	1.03
α-Ketoglutaric acid 7.12	at 37° for 15 min.	for 18-24 hrs	7.06

* All figures are expressed in terms of α-ketoglutaric acid.

Recovery Test of α-Ketoglutaric Acid Added to Blood and Urine—The determination of α-ketoglutaric acid in blood and urine

was carried out, and the recovery of the added amount was examined. The results are shown in Table VI and VII. The recovery was above 98.4% in most cases of blood and above 96.6% in all the cases of urine.

TABLE VI

Blood	α-Ketoglutaric acid added (mg)	Found (mg./dl)	Recovery (%)
Human I (1 : 2)	— 3.56	0.09 3.63	99.4
Human II (1 : 2)	— 3.56	0.04 3.56	99.0
Human III (1 : 2)	— 3.56	0.52 4.04	98.9
Rabbit I (1 : 2)	— 3.56	0.26 3.76	98.4
Rabbit II (1 : 2)	— 3.56	0.56 4.08	98.9
Rabbit III (1 : 2)	— 3.56	0.67 4.17	98.4
Rat I (1 : 2)	— 3.56	0.28 3.78	98.4
Rat II (1 : 2)	— 3.56	0.50 3.93	96.4
Rat III (1 : 2)	— 3.56	0.02 3.52	98.4

α-Ketoglutaric and Pyruvic Acids in Human Blood—The α-ketoglutaric and pyruvic acids in the blood of 13 healthy men (students and workers in the laboratory), 18 to 34 years of age, were determined (Table VIII). The blood was withdrawn with minimum stasis from cubital veins of the persons sitting on a chair for 2 hours and more than 3 hours after the last meal. Pyruvic acid was determined by the modification (9) of the method of Friedeman and Haugen (6). The average value of α-ketoglutaric acid was 0.63 mg./dl, and that of pyruvic acid was 0.88 mg./dl.

TABLE VII

Urine	α-Ketoglutaric acid added (mg.)	Found (mg./dl)	Recovery (%)
Human I (1 : 2)	—	1.14	
	3.56	4.62	98.3
Human II (1 : 2)	—	1.10	
	3.56	4 61	98.6
Human III (1 : 2)	—	1.40	
	3.56	4.88	97.9
Rabbit I (1 : 2)	—	2.50	
	3.56	5.99	97.9
Rabbit II (1 : 2)	—	1.23	
	3.56	4.72	98.0
Rabbit III (1 : 2)	—	2.34	
	3.56	5.84	98.4
Rat I (1 : 4)	—	5.39	
	0.89	6.25	96.6
Rat II (1 : 4)	—	5.02	
	0.89	5.88	96.6
Rat III (1 : 4)	—	5.25	
	0.89	6.12	97.8

TABLE VIII

Name	Age	α-Ketoglutaric acid in blood (mg./dl)	Pyruvic acid in blood (mg./dl)
H. M.	18	0.69	0.83
N. S.	33	0.67	0.84
S. H.	34	0.65	0.59
T. K.	34	0.72	0.81
H. M.	27	0.52	1.01
T. O.	24	0.69	0.98
T. N.	22	0.65	0.98
T. H.	25	0.48	0.94
K. N.	25	0.60	0.94
T. H.	25	0.69	1.00

T. O.	24	0.73	0.70
K. N.	25	0.52	0.80
T. N.	22	0.63	1.02
Mean		0.63±0.023 *	0.88±0.040 *

* mean square error

α-Ketoglutaric and Pyruvic Acids in Human Urine—The α-ketoglutaric and pyruvic acids in the urines of 10 healthy men (students and workers in the laboratory), 20 to 41 years of age, were determined (Table IX). The average of α-ketoglutarate was 1.10 mg./dl in the concentration and 30 mg. in the daily excretion. The average of pyruvate was 0.91 mg/dl in the concentration and 21 mg. in the daily excretion.

TABLE IX

Name	Age	Urine quantity (ml.)	α-Ketoglutaric acid		Pyruvic acid	
			Concentration (mg./dl)	Daily excretion (mg.)	Concentration (mg./dl)	Daily excretion (mg.)
T. S.	29	2000	1.62	32	0.91	18
S. K.	41	2900	1.77	59	0.66	19
K. I.	20	2120	1.68	23	0.99	21
B. U.	28	2200	0.86	19	1.44	32
I. G.	24	1800	1.17	21	1.65	30
S. H.	34	2600	1.11	29	0.69	18
K. Y.	23	2500	1.00	25	0 59	15
T. S.	29	1900	1.25	24	0.88	17
B. U.	28	2620	1 02	27	0.71	19
S. H.	34	3360	0.13	44	0.60	20
Mean		2400± 127.1 *	1.10±0.117 *	30±3.6 *	0.91±0.109 *	21±1.7 *

* mean square error

Summary

The new method of the determination of α-ketoglutaric acid in blood and urine is offered which is sufficiently sensitive and specific. It is based upon the difference of the formation rate and solubility of 2, 4-dinitrophenylhydrazones of α-ketonic acids and the ease of decomposition of β-ketonic acids by aminoantipyrine. The final reading was made colorimetrically, adding alkali to the hydrazone of α-ketoglutaric acid. The extract with xylene in this method can be used for the determination of pyruvic acid. Thus it is possible to determine the amount of pyruvic acid and of α-ketoglutaric acid by using the same sample.

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The Journal of Biochemistry, Vol. 37, No. 4.

ENZYMATIC TRANSFER OF GLUCOSE
II. IDENTITY OF GLUCOTRANSFERASE AND β-GLUCOSIDASE

By

KATSUO TAKANO and TOMOO MIWA

(*From the Botanical Institute, Tokyo Bunrika University, Tokyo*)

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The enzyme, first discovered by J. Rabaté, (1) which transfers β-glucoside residue of aryl-β-glucoside to alcohol was further investigated and named glucotransferase (2). Enzyme preparations which had been obtained from green leaves of various plants, exhibited invariably both transferring and hydrolyzing activity toward phenol- or *p*-nitrophenol-β-D-glucoside. At that time it was tentatively suggested that glucotransferase might be a particular enzyme distinct from usual β-glucosidase, the hydrolyzing action of the enzyme preparation being due to the contamination of β-glucodidase.

Meanwhile it happened unexpectedly that apricot emulsin as well as highly purified β-glucosidase preparations from *Aspergillus niger* exhibited marked activity of glucotransferase. These observations naturally necessitated the reexamination of the nature of glucotransferase. The experimental findings described below seem to favor the view that glucotransferase and β-glucosidase might be regarded as identical.

Experimental

For quantitative test *p*-nitrophenol-β-glucoside was used as glucose donor, and methanol or *n*-butanol as its acceptor. By the action of enzyme *p*-nitrophenol is liberated whereas glucose is partly set free, partly accepted by alcohol forming alkyl-β-glucoside. Liberated *p*-nitrophenol was determined colorimetrically and free

glucose reductometrically. The degree of glucose transfer, which is expressed as follows,

$$\frac{[p\text{-nitrophenol liberated}]-[\text{glucose liberated}]}{[p\text{-nitrophenol liberated}]}\times 100$$

can be obtained by comparison of these values.

Experiments with Apricot Emulsin

Enzyme preparations:

Apricot emulsin I. The enzyme solution was obtained by extraction of defatted powder of apricot seeds with water and freed from reducing substances by dialysis ($f_{ph.-\beta-gl.}=0.017$) (3).

Apricot emulsin II. It was prepared after the method of B. Helferich and co-workers (4), its activity corresponding to that of Helferich's "Rohferment" ($f_{ph.-\beta-gl.}=1.9$).

Apricot emulsin III. Praparation II was further purified by fractional precipitation with silver oxide after Helferich (5) ($f_{ph.-\beta-gl.}=3.06$).

By the use of these emulsin preparations the glucoside residue of p-nitrophenol-β-glucoside was transferred to methanol or n-butanol.

Reaction mixture:

0.048 M	p-Nitrophenol-β-glucoside	2.0 ml.
0.2 M	Acetate buffer pH 4.8	2.0 ml.
5 M	Methanol or 2.5 M n-butanol	2.0 ml.
Apricot emulsin solution		4.0 ml.

Mixture without acceptor contained 2.0 ml. water in place of alcohol.

Temp. 30°

TABLE I

Enzyme preparation f	Acceptor	Reaction time min.	Substrate cleaved: p-Nitrophenol liberated %	Substrate cleaved: Glucose liberated %	Glucose transferred to alcohol, % of p-nitrophenol-β-glucoside cleaved
0.017	Methanol	60	42	31.5	25
		120	57	41.2	28
		240	68	49.2	27
	n-Butanol	60	18	11.1	39
		120	32	19.2	40
		240	48	26.2	45
	None	60	45	48.0	4.4
		120	60	59.8	0.3
		240	70	67.9	3.0
1.9	Methanol	90	53	42.1	21
		120	65	46.8	28
		150	70	56.8	20
	n-Butanol	90	50	35.2	30
		120	65	42.1	35
		150	70	44.5	36
	None	90	55	55.2	−0.4
		120	65	63.7	2.0
		150	70	70.0	0.0
3.06	Methanol	40	24	17.7	26
		90	37	28.5	23
		150	47	33.0	30
	n-Butanol	40	12	8.2	32
		90	27	15.2	44
		150	40	22.8	43
	None	40	25	23.6	5.6
		90	37	36.6	0.2
		150	47	47.0	0.0

The results of Table I indicate that hydrolysis and transfer action proceeded nearly parallel with preparations of different purity. No evidences for the distinction of transferase and hydrolase were observed.

Formation of β-Methylglucoside by the Enzymatic Transfer of Glucose

Direct evidence for glucose transfer by apricot emulsin was furnished by the isolation of β-methylglucoside. As glucose donor *o*-cresol-β-glucoside and as acceptor methanol was used, since this substrate is extremely rapidly and β-methylglucoside is slowly hydrolyzed by emulsin, favoring the accumulation of the transfer product. 2.0 g. *o*-cresol-β glucoside were dissolved in 100 ml. 5 per cent methanol, to which 0.1 g. apricot emulsin ($f_{ph.-\beta-gl.} = 1.9$) was added and made to pH 4.8 with acetic acid. After incubation at 30° for 5 hrs., at the end of which time *o*-cresol-β-glucoside had been completely split, the reaction mixture was heated to boiling and filtered. *o*-Cresol was removed by repeated extraction with ether and free glucose by fermentation with brewer's yeast. The solution was heated again and the clear filtrate was evaporated to dryness under reduced pressure. The residue was extracted with 10 ml. hot ethanol, which, on keeping in ice box, deposited crystals. They were filtered, washed with ethanol and recrystallized from absolute ethanol. The product, which weighed 0.2 g. was identified with β-methylglucoside by melting point of 110° and specific rotation of $[\alpha]_D^{20} = -33.8°$ (H_2O).

Experiments with β-Glucosidase Preparations of Aspergillus Niger (6)

The mold was cultured in Henneberg's solution with sucrose and peptone as carbon and nitrogen source respectively. Grown mold was harvested at the beginning of spore formation and dried.

Enzyme was extracted with water and purified by repeated precipitation with tannin. The activity of the preparations employed in the present experiments were; $f_{ph.-\beta-gl.} = 4.3$, 248 and 510. Conditions of the enzyme reaction were the same as with apricot emulsin.

TABLE II

Enzyme preparation f	Acceptor	Reaction time min.	Substrate cleaved		Glucose transferred to alcohol, % of p-nitrophenol-β-glucoside cleaved
			p-Nitrophenol liberated %	Glucose liberated %	
4.3	Methanol	60	16	11.0	31
		120	28	20.6	27
		210	50	34.5	31
	n-Butanol	60	13	11.0	15
		120	25	21.4	14
		210	45	38.4	15
	None	60	10	11.0	−1.0
		120	20	21.4	−0.7
		210	35	33.6	4.0
248	Methanol	60	42	28.4	32
		90	65	41.4	36
		160	87	58.3	33
	n-Butanol	60	40	35.2	12
		90	62	50.2	19
		160	87	73.6	15
	None	60	32	32.2	−0.6
		90	48	46.0	4.2
		160	83	83.0	0.0
510	Methanol	120	38	26.6	30
		180	60	37.4	38
		320	80	54.3	32
	n-Butanol	120	35	31.5	10
		180	60	49.2	18
		320	80	70.0	13
	None	120	30	29.3	2.3
		180	45	45.3	−0.6
		320	80	77.8	2.7

As shown in the above table, all β-glucosidase preparations of Aspergillus niger exhibited marked activity of glucotransferase. The behavior of the enzyme toward methanol and n-butanol, as expressed by the degree of glucose transfer, remained unaltered irrespective of the purity of enzyme preparation. These findings may be

taken to indicate that one and the same enzyme concerns both in glucose transfer and hydrolysis.

Experiment with β-Glucosidase Preparation of Penicillium Chrysogenum

This mold is the one used in the manufacture of penicillin. It is cultured in a large scale in tank.* Enzyme preparation was obtained in a similar way as those of *Aspergillus niger* ($f_{ph.-\beta-gl.}$ $=10$). Conditions of the reaction were the same as with apricot emulsin.

TABLE III

Acceptor	Reaction time min.	Substrate cleaved		Glucose transferred to alcohl, % of *p*-nitrophenol-β-glucoside cleaved
		p-Nitrophenol liberated %	Glucose liberated %	
Methanol	30 80	13 32	11.0 26.7	15 16
n-Butanol	30 80	25 55	12.1 28.5	52 53
None	30 80	10 28	11.1 28.0	−1.1 0.0

These results indicate clearly that the β-glucosidase preparation of *Penicillium* had strong activity of glucotranferase.

Glucose Transfer not a Reversal of Hydrolysis

In a previous paper (2) we have indicatied that the enzymatic formation of alkyl-glucoside from aryl-β-glucosid and alcohol under conditions of our experiments cannot be regarded as a simple reversal of hydolysis, which, however, is known to take place by the action of emulsin in higher concentraton of both alcohol and glucose.

* Dried mold was supplied from Nippon Penicillin Co. Ltd. through the courtesy of Mr. T. Kondo, to whom we wish to express our gratitude.

By the action of each of the enzyme preparations used in the above experiments upon dilute solution of glucose (0.0096 M) and methanol (2 M), no decrease of free glucose could be ascertained, indicating that the glucoside formation had not taken place (Table IV).

TABLE IV

Reaction mixture:	0.048 M D-glucose	2.0 ml.
	0.2 M Acetate buffer	2.0 ml.
	Enzyme solution	4.0 ml.
	10 M Methanol	2.0 ml.
	Temp. 30°	

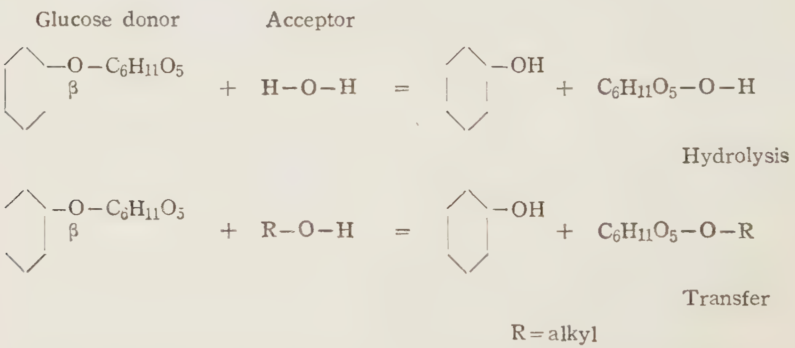
Enzyme	Reaction time hr.	Free glucose mg.
Apricot emulsin $f_{ph.-\beta-gl.}=1.9$	0	17.3
	10	17.1
	24	17.4
Asp. niger $f_{ph.-\beta-gl.}=4.3$	0	17.3
	10	17.2
	24	17.3
Pen. chrysogenum $f_{ph.-\beta-gl.}=10$	0	17.3
	10	17.3
	24	17.2

DISCUSSION

In a previous communication (2) it was stated that the transfer of glucose from aryl-glucoside to alcohol might be catalyzed by a specific enzyme, named glucotransferase, which is present in green leaves of various plants and probably differs from the usual β-

glucosidase. Present investigation revealed that this transferase activity is found in every β-glucosidase preparation examined. In β-glucosidase preparations of different purity (Wertigkeit) there existed an approximate parallelism between the activity of hydrolysis and that of transfer (Table I and II). No tendency for the separation of glucotransferase and β-glucosidase could be recognized in the purification of enzyme preparations. On the basis of these findings the writers now hold a view that glucotransferase activity is an inherent character of β-glucosidase, namely gucotransferase and β-glucosidase may be idenltical.

Fom this point of view the relationship between hydrolysis and transfer action of β-glucosidase may be schematically represented as follows:



It will be seen that where aryl-β-glucoside is used as substrate for β-glucosidase, both water and alcohol are able to act as glucose acceptor, forming free glucose and alkyl-β-glucoside respectively as cleavage product. The enzyme of green leaves, in which glucotransferase activity was recognized for the first time, may also be in all probability nothing but an usual β-glucosidase.

As shown in the above experiments the enzymatic transfer of glucose proceeds without mediation of phosphate, so the mechanism of the reaction may be different from that catalyzed by transglucosidase of Doudoroff and associates (7), which works only under

intervention of phosphate. In this respect our enzyme appears to belong to the same category as that of phosphotransferase of Axelrod (8).

The data in Table I, II and III indicate that the behavior of β-glucosidase toward glucose acceptor differs with the source of the enzyme preparation; e.g. with apricot emulsin and the enzyme of Penicillium, *n*-butanol is an efficient acceptor as compared with methanol, while with the enzyme of Aspergillus the reverse is the case. These facts may be understood as difference in the relative acceptor specificity of the enzyme, which is generally recognized in case of dehydrogenases (H-transferases).

It the foregoing paper the observations were described that enzymatically combined glucose was again released with long reaction time and this was taken at that time as an evidence for the separate existence of glucotransferase and β-glucosidase. In the light of the unitary concept, however, this phenomenon can be interpreted without difficulty. Namely, as the formation of methyl-glucoside takes place only in the presence of aryl-glucoside, so the decrease of the former must result after the glucose donor is consumed completely.

As stated in the previous communication (2) the transfer and hydrolysis action of leaf enzymes behaved somewhat independently in the course of heat inactivation and this was considered as favoring the view of the dual nature of glucotransferase and β-glucosidase, In view of the observations that the relative specificity of β-glucosidase may be sometimes modified by the action of heat on enzyme protein (9), it appears rather premature to conclude that glucotransferase and β-glucosidase are different enzymes.

In mold enzymes a tendency was noticed that when alcohol was added to the reaction mixture the rate of substrate cleavage was enhanced as compared with its absence. This may be ascribed to the high affinity of alcohol to enzyme substrate complex and also to the high decomposition rate of the formed compound.

Summary

1). Enzymatic transfer of glucose from aryl-β-glucoside to alcohol, which had been observed with enzyme preparations from green leaves, was also recognized in usual β-glucosidase preparations, such as apricot emulsin and enzymes of mold fungi, *Aspergillus niger* and *Penicillium chrysogenum.*

2). From the parallelism between transferring and hydrolyzing activity in enzyme preparations of different purity, it is suggested that the enzymes responsible for transfer and hydrolysis are identical.

3). The hydrolysis and transfer action of β-glucodidase may be interpreted in terms of acceptor difference; when water acts as glucose acceptor the hydrolysis results, whereas the use of alcohol leads to the formation of alkyl-β-glucoside.

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DETERMINATION OF FREE AND ESTERIFIED RIBOFLAVIN BY LUMIFLAVIN METHOD.

By

AKIJI FUJITA and KWANJI MATSUURA.

(*From the Biochemical Laboratory, the Kitcsato Institute, Tokyo*)

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The lumiflavin method, which was developed by Warburg and Christian (1) for the first time in studies of yellow oxidation enzyme, has been studied and improved by Kuhn (2), Reindel (3) and Koyanagi (4). But until now it has made no remarkable progress, and the direct fluorometary has been commonly used by many investigators such as Euler (5), Koschara (6), Hodson (7) and Bessey (8) (9). After the investigation of many fundamental conditions and the comparison of both methods in estimating the vitamin contents of many animal and vegetable tissues, we have come to the conclusion that the lumiflavin method carried on under adequte conditions is most specific and reliable, free from blank fluorescence, and recoveries are always practically complete. The direct fluorometry is, on the contrary, not always specific enough and not always free from blank fluorescence. The adsorbents, which are absolutely necessary in the case of many biological materials for the removal of interfering substances, are found, in no case as far as we tested, to be able to adsorb and elute riboflavin completely, recoveries being never satisfactory.

It has been further demonstrated that the esterified riboflavin including flavin adenine dinucleotide (FAD) and flavin phosphoric acid (FMN) are equally converted to lumiflavin just as riboflavin (B_2) itself, so that preliminary enzymatic hydrolysis is not necessary, in this case, for estimating total B_2. Since esterified B_2 can be distinguished from B_2 on the basis of its distribution coefficient between benzyl alcohol and aqueous solution (Emmerie (10)), ready

analytica means are available for measuring free and esterified B_2 in biological materials.

A description given below is of the improved micro-lumiflavin method for measuring free and esterified B_2 in biological materials, to which we have reached after many fundamental researches and which has been proved to be satisfactory after numerous estimations.

Principles

Tissues are ground after heating at 80° to destroy phosphatase activity for decomposing FAD, and are extracted with water at 80° completely. It is made to pH 4.5, whereby protein is largely precipitated, and the supernatant after centrifugation becomes clear. An aliquot is photolyzed, and the total B_2 is estimated. Another aliquot is extracted two or three times with an equal volume of benzyl alcohol, whereby the free B_2 is practically completely extracted. The remaining extract is photolyzed and the esterified B_2 is estimated, correction being made due to extracted loss.

After photolysis B_2 becomes extractable with chloroform in acid solution and such property is observed only in B_2 or its derivatives among many natural substances. The lumiflavin method is, therefore, very specific for B_2. The conversion to lumiflavin is not complete, but about 80%. But under adequate conditions, the percentage of conversion remains constant independently of the amount of B_2, so that it can be estimated quantitatively, if the standard and the test solutions are photolyzed under the same conditions.

Materials

1. 2 *N* NaOH
2. 1 *N* NaOH
3. Choroform
4. Glacial acetic acid.
5. 2 *N* H_2SO_4.
6. 10% Trichloroacetic acid (TCA).

7. 4% Potassium permanganate solution (Mn)
8. 3% Hydrogen peroxide solution.
9. Benzyl alcohol, redistilled *in vacuo* and saturated with water. It must be clear and colorless and free from fluorescence.
10. Acetate buffer at pH 4.5.
    62 ml. of $N/10$ acetic acid and 38 ml. of $N/10$ sodium acetate are mixed.
11. Standard B_2 solution.
    Aqueous solution of 4 mg. of B_2 and 2 drops of glacial acetic acid in 100 ml.
12. Mercury vapor lamp with adequate filter.
13. Komagome capillary pipettes with rubber cups.
    Except for pipettes for removing chloroform, we use a special capillary pipette for removing benzyl alcohol, the capillary end of which is strongly extended to about 1 mm. in calibre.
14. Centrifuge-tubes with glass-stopper, 2.5×9 cm., about 25 ml. capacity, used for photolysis as well as centrifugation.
15. Microburette for standard chloroform solution of lumiflavin, always coverd with water about 1 cm. high above chloroform solution for preventing evaporation of chloroform.
16. Test-tubes, free from fluorescence.
17. Photolysis apparatus.
    Thermostat about $23\times21\times34$ cm., filled with water. Front wall is made of glass. In front of the wall, two 100—200

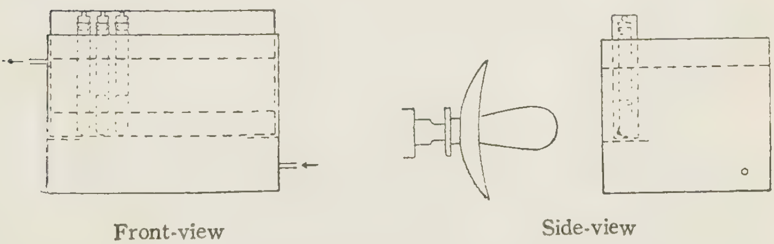

Fig. 1. Photolysis apparatus

watt-electric lamps are placed at the distance of about 20 cm. as shown in Fig. 1. Thermostat is kept at 30°

Procedure

Determination of Esterified and Total B_2 in Tissues

(1) Extraction

p g. (usually 1–10 g.) of tissue are cut with a sharp razor in pieces about (0.5 cm³.) and thrown into water in a test-tube, which has been heated in advance to 90°. The tube is allowed to stand at 80° for 15 minutes in a water bath. The pieces are ground, the extract is quantitatively, added and water is added to an adequate volume. After heating at 80° for 5 minutes the whole is cooled and made to pH 4.5 with 1 N HCl, water being added to keep a constant volume (usually 20–40 ml.). After heating at 80° for 5 minutes, the solution is cooled and centrifuged. The supernatant A $(1:v)$ is used for further treatment.

Remarks: (a) When the extract in 0.25 N H_2SO_4 is heated at 80°, the esterified B_2 is decomposed gradually. The extraction of B_2 in tissues with water under the described condition is complete practically. At pH 4.5 protein is largely precipitated and the supernatant becomes almost transparent. After heating the solution of pH 4.5 at 80° for 15 minutes, no hydrolysis of esterified B_2 takes place. (b) The photolysis of free and esterified B_2 takes place equally under the described conditions. The enzymatic hydrolysis of the esterified B_2 is, therefore, unnecessary for estimating total B_2.

(2) Photolysis

I. Determination of Total B_2

a ml. of the supernatant A is diluted with water to 10 ml. and is mixed with 10 ml. of 1 N NaOH. 5 ml. is transferred to each of the three small covered centrifuge-tubes, one of which is used for recovery test, with the addition of 0.3 μg. of B_2. The remaining

two are used for main and blank tests, respectively. The blank test is kept in the dark. After photolysis for one hour, 0.3 ml. of glacial acetic acid and 5 ml. of chlorform are added to each tube. After vigorous shaking they are centrifuged. The supernatant aqueous layer being drawn off previously c ml. (usually 4 ml.) of the chloroform layer is taken for determination.

II. Determination of Esterified B_2

b ml. of the supernatant A (containing about 1 μg. of total B_2) is transferred to each of the covered centrifuge-tubes, water being added to 12 ml. Thorough extraction of free B_2 is accomplished by a vigorous agitation with an equal volume of benzyl alcohol saturated with water. After centrifugation 10 ml. of the aqueous layer is transferred to another covered centrifuge-tube and is shaken vigorously with an equal volume of benzyl alcohol saturated with water. After centrifugation the benzyl alcohol layer is drawn off as completely as possible. Usually twice extractions are neccessary for complete removal of free B_2. When there is a large amount of B_2 three times extractions are necessary for its complete removal. 4 ml. of the aqueous layer is transferred to each of the two centrifuge-tubes, one of which is used for blank test, photolysis being omitted. Each tube is mixed with 2 ml. of 2 N NaOH. After photolysis for one hour 0.3 ml. of glacial acetic acid is added and extracted vigorously with 6 ml. of chloroform. After centrifugation c ml. (usually 5 ml.) of the chloroform layer are taken for determination.

Remarks: (a) When there is much blank fluorescence, manganese treatment after the addition of glacial acetic acid is to be given in the following way.

0.5–1 ml. of 4 % Mn is added and within 2 minutes the necessary amount of H_2O_2 solution is added to destroy the excessive Mn only. (b) each 12 ml. of extract and benzyl alcohol are shaken at the first extraction in order to avoid intermixing of the intermediary zone formed at the interface of both layers in the case of tissues.

(3) Estimation and Calculation

The standard B_2 solution is added to the blank test till the fluorescence matches the main test (m) and further added to match the recovery test (m'), Total B_2 is calculated by the following formula.

$$x=\frac{m\times 0.3}{m'-m}\times\frac{20}{5}\times\frac{v}{a}\times 100=\frac{120\,mv}{(m'-m)\,a}\,(\mu\text{g.}/100\text{ g.})$$

Without recovery test:

$$x=m\times\frac{5}{c}\times\frac{20}{5}\times\frac{v}{a}\times 100=\frac{2000\,mv}{ac}\,(\mu\text{g.}/100\text{ g.})$$

Esterified B_2:

$$x=\frac{m}{k}\times\frac{6}{c}\times\frac{12}{4}\times\frac{v}{b}\times 100=\frac{1800\,mv}{kbc}\,(\mu\text{g.}/100\text{ g.})$$

The values of k are found in Table I.

TABLE I

Number of extractions (t)	$k=0.96^t$
1	0.96
2	0.92
3	0.89
4	0.85

Remarks: (a) The distribution coefficient of FAD, and FMN are not the same, but the ratio of the distribution in aqueous layer under above conditions remains practically the same, namely 0.96. (See the part on experiment below).

(b) Shaking must be vigorous enough to reach an equilibrium state of distribution. In tests with solution containing 1 μg. of pure B_2 the distributed amount in the aqueous layer must be 0.23, 0.05 and 0.01, respectively after one, two, and three times extractions, respectively.

(c) Recovery is practically complete, usually 98-100 %.

Examples.

Beef liver (commercial).

(Total B_2).

$a=1, \quad v=40, \quad m=0.16, \quad m'=0.40, \quad m'-m=0.24,$

$$x=\frac{120\times 0.16}{0.24}\times 40=3200\ (\mu g./100\,g.)$$

Without recovery test:

$$x=2000\times\frac{0.16\times 40}{1\times 4}=3200\ (\mu g./100\,g.)$$

(Esterified B_2)

$b=6, \quad m=0.42, \quad c=2.$

$$x=\frac{1800\times 0.42\times 40}{0.85\times 6\times 2}=2960\ (\mu g./100\,g.)$$

$$\frac{\text{Ester}}{\text{Total}}=\frac{2960}{3200}\times 100=92.5\ \%$$

$$\text{Therefore}\ \frac{\text{Free}}{\text{Total}}=100-92.5=7.5\ \%$$

Determination of Free and Esterified Riboflavin in Blood

(1) Extraction

0.6 ml. of 1 N HCl is added to 6 ml. of blood and water is added to 32 ml. After heating at 80° for 15 minutes it is cooled and is mixed with 8 ml. of 10% TCA. After centrifugation the supernatant A is taken for determination.

(2) Photolysis

I. Determination of Total B_2

4 ml. of 2 N NaOH is added to 8 ml. of A. After photolysis 0.6 ml. of glacial acetic acid and 6 ml. of chloroform are added. After thorough shaking 5 ml. is taken for estimation. For a blank test the reagents alone are used.

II. Determination of Esterifiied B_2

8 ml. of A is transferred to each of two centrifuge-tubes, 1 ml.

of acetate buffer (pH 4.5) is added and its pH is made to 4.5 with 1 N NaOH. Water is added to 10 ml. They are thoroughly shaken with an equal volume of benzyl alcohol saturated with water. 8 ml. of the supernatant are mixed with 4 ml. of 2 N NaOH and photolyzed, while a blank test is placed in the dark. After addition of 0.6 ml. of glacial acetic acid and thorough extraction with 6 ml. of chloroform and centrifugation, 5 ml. of chloroform layer are taken for estimation.

(3) Calculation

When the recovery of the added B_2 is R %, the B_2 content can be calculated by the following formula.

(Total B_2)

$$x = m \times \frac{100}{R} \times \frac{6}{5} \times \frac{40}{8} \times \frac{100}{6} = 10{,}000 \times \frac{m}{R} \quad \mu\text{g./100 g.)}$$

(Esterified B_2)

$$x = m \times \frac{100}{R} \times \frac{6}{5} \times \frac{10}{8} \times \frac{40}{8} \times \frac{100}{6} \times \frac{1}{0.96} = 13{,}000 \times \frac{m}{R}$$

(μg./100 g.)

Remarks

(a) The recoveries of the added B_2 are found to be 94–100 %. The shortage comes in the deproteinization procedure. The added B_2 to the deproteinized filtrate can be almost completely recovered after photolysis. The addition of TCA should be made dropwise and the solution must be thorougly shaken.

(b) The strength of blank fluorescence without photolysis is always the same as that of reagent blank. The recovery is practically constant independently of the amount of B_2. Therefore, omitting tne blank and recovery tests, we can estimate the B_2 content in the following way using only 2 ml. of blood; To 2 ml. of blood 10 ml. of water are added. After heating at 80° for 15 minutes it is cooled. 3.0 ml. of 10 % TCA are added, and the whole is treated in the same way as described above.

(Total B_2)

$$x = m \times \frac{100}{R} \times \frac{6}{5} \times \frac{15}{8} \times \frac{100}{2} = 11{,}300 \times \frac{m}{R} \quad (\mu g./100 \text{ ml.})$$

Examples. $m = 0.055$. $R = 94$ %.

(Total B_2)

$$x = 0.055 \times \frac{100}{94} \times 100 = 5.7 \ (\mu g./100 \text{ ml.})$$

(Esterified B_2) $m = 0.04$

$$x = 0.04 \times \frac{100}{94} \times 130 = 5.5 \ (\mu g./100 \text{ ml.})$$

$$\frac{\text{Ester}}{\text{Total}} = \frac{5.5}{5.7} \times 100 = 97 \ \%$$

$$\text{Therefore } \frac{\text{Free}}{\text{Total}} = 100 - 97 = 3 \ \%$$

Determination of Free and Esterified Riboflavin in Urine.

As urine contains much blank fluorescence, it is necessary to destroy it after photolysis by permanganate and the excess permanganate is removed by H_2O_2.

(1) Extraction and photolysis.

To *a* ml. (usually 3–5 ml.) of urine 5—*a* ml. of water are added. To the recovery test 1 μg. of B_2 is added. After mixing with 5 ml. of 1 *N* NaOH it is centrifuged. 5 ml. of the supernatant are transferred to each of the three covered test-tubes, one of which is used as a blank test and is kept in the dark. After photolysis for one hour, 0.4 ml. of glacial acetic acid is added to each tube. 1.0 ml. of 4 % Mn solution being added, the excessive Mn is decolorized at once with 3 % H_2O_2. After being vigorously shaken with 5.0 ml. of chloroform it is centrifuged, 4 ml. of the supernatant being used for estimation.

Remarks. (a) 1.5 ml. of Mn can equally be used. In proportion to the existing amount of substances, which consume Mn, the adequate amount of Mn should be used.

(b) After addition of Mn, H_2O_2 must be added at once. Within

2 minutes the recovery was found to be practically constant, while it was 85 % after 5 minutes and 45 % after 10 minutes.

(c) For the estimation of esterified B_2, the urine is made to pH 4.5 and is treated with benzyl alcohol as described above.

(2) Calcutation.

Total B_2 is calculated by the following formula.

$$x = \frac{m}{m'-m} \times \frac{100}{a} \quad (\mu g./100\ ml.)$$

$$\text{Recovery:} \quad \frac{m'-m}{1 \times \frac{5}{10} \times \frac{4}{5}} \times 100 = 250\ (m'-m) \quad \%$$

Without recovery test:

$$x = m \times \frac{5}{4} \times \frac{10}{5} \times \frac{100}{a} = 250 \times \frac{m}{a} \quad (\mu g./100\ ml.)$$

Remarks.

As the distribution of lumiflavin in chloroform layer is practically complete, slight differences in volume ratios of aqueous to chloroform layer have scarcely any effect on the results.

Example. Adult male subject, 30 years old,

$$a = 3.0, \quad m = 0.090, \quad m' = 0.490, \quad m' - m = 0.400$$

$$x = \frac{0.09}{0.40} \times \frac{100}{3} = 7.5 \ (\mu g./100\ g.)$$

Recovery: $250 \times 0.4 = 100 \quad \%$

Without recovery test:

$$x = 250 \times \frac{0.09}{3} = 7.5 \ (\mu g./100\ g.)$$

EXPERIMENTAL

1. *Distribution of Free and Esterified B_2 between Water and Benzyl Alcohol*

The distribution coefficients for B_2, FMN and FAD between benzyl alcohol and the neutralized TCA extract are, according to Burch (9), 3.8, 0.02, and 0.01, respectively, fromw hich the distributed amount in the aqueous layer after shaking with equal volumes of

water and benzyl alcohol can be calculated, values of 0.203, 0.98, and 0.99, respectively being obtained. The latter two are practically the same. When the B_2 in 0.01 *M* buffer (pH 4.5) and an equal volume of benzyl alcohol saturated with water are shaken vigorously, the distributed amount of B_2 in the aqueous layer is 0.23. The extract of beef liver (pH 4.5) containg about 1 μg. of B_2 are shaken with an equal volume of benzyl alcohol saturated with water three times. The free B_2 is practically completely removed. Then before and after extraction with an equal volume of benzyl alcohol saturated with water, the B_2 contents are estimated. The distributed amount in the aqueous layer was found to be 0.96. The same tests were also performed with extracts of beef retina, chorioidea, rabbit spleen, kidney, dried sardine, green leaves, green peas, cucumber, rice bran, brewers' yeast and seaweeds. The results were all practically the same and found to be 0.96.

2. *Extraction of Total B_2 from Tissues*

Beef liver was cut in small pieces, heated in water at 80° for 15 minutes, ground well and treated in the following way: (*1*) After heating at 80° for 5 minutes pH was made to 4.5 and heated at 80° for 5 minutes. (*2*) pH is made at once to 4.5 and heated at 80° for 5 minutes. (*3*) The extract is made 0.25 *N* H_2SO_4 in final concentration and heated at 80° for 15 minutes. (*4*) The same as (*3*) except for heating at 80° for 60 minutes. (*5*) The extract is made 1 *N* H_2SO_4 in final concentration and heated at 80° for 60 minutes.

As shown Table II, in the results of the extraction is complete under the described condition, and the filtrate is at the same time the most clear and transparent.

The same results were also obtained in brewers' yeast, spinach, stone-leek and sweet potato.

TABLE II

Effect of Various Treatments upon the Extraction of B_2.

− transparent, + turbid, ± slightly turbid.

Treatment	Total B_2 concentration (μg./100 g.)	Turbidity of Filtrate
(1)	3100	=
(2)	2900	=
(3)	3100	+
(4)	3100	+
(5)	3100	±

3. *Effect of Heating upon the Esterified B_2*

In order to know, whether the esterified B_2 may decompose under the described conditions of extraction, the contents of esterified B_2 were successively estimated and we found that the contents of esterified B_2 remain unchanged when heated at 80° for 15 minutes.

4. *Distribution of Free and Esterified B_2 in Animal aud Vegetable Tissues*

According to experiments of our laboratory (Sakamoto (10)) on the paper partition chromatography the esterified B_2 in animal tissues exists for the most part in the form of FAD and trace or at most 10 % of the total B_2 in the form of FNN and no free B_2 is detectable except in hen's egg and cow's milk, so long as the extraction of B_2 is performed under adequate conditions. We examined the distribution of free and esterified B_2 in animal and vegetable tissues, the results of which are shown in Table III. From the table we see that in animal tissues B_2 exists largely in esterified form, except in urine, feces, placenta, hen's egg and animal milk. On the contrary B_2 is largely in the esterified form in human milk. In green leaves about 50–60 % of total B_2 are found to exist in esterified form.

TABLE III

Distribution of Free and Esterified B_2 in Biological Tissues.

Species	Tissue	B_2 content (μg./100 g.) Ester	Total	$\frac{\text{Ester}}{\text{Total}}$ (%)
Cow	Liver	3080	3200	96
	Milk	21	100	21
Goat	Milk	36	320	11
Man	Blood	5.6	5.7	98
	Urine	2.7	14	20
	Feces	264	710	37
	Milk	42	44	96
	Placenta	31	125	25
	Umbilical cord	14	21	66
Hen	Egg white	0	280	0
	Egg yolk	0	400	0
Rice bran		209	250	84
Sweet potato	Young leaves	362	800	45
	Old leaves	543	920	59
Cabbage	Interior	14	42	31
	Exterior	18	31	58
Green peas		94	135	70
Cucumber		30	31	95
Brewers' yeast		870	900	97

Summary

1. The improved lumiflavin method for estimating free and esterified B_2 is described in detail.

2. The lumiflavin method is exact, reproducible and specific for B_2, the recoveries being always practically cemplete and the blank fluorescence can be completely removed by permanganate treatment.

3. Free and esterified B_2 are equally converted to lumiflavin without preliminary enzymatic hydrolysis.

4. The distribution of free and esterified B_2 in animal and vegetable tissues is reported.

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The Journal of Biochemistry, Vol. 37, No. 4.

ENZYMIC SYNTHESIS OF ADENINE-FLAVINE-DINUCLEOTIDE.

By

KATASHI MAKINO, FUMIO ITOH and KIYOHIKO OHTA

(From the Department of Biochemistry, Kumamoto Medical College, Kumamoto)

(Received for publication, August 15, 1950)

Knowledge of the mechanism of enzymic synthesis of adenine-flavine-dinucleotide is lacking. We (1) have found that pig acetone kidney powder contains a thermolabile enzyme system capable of the synthesis of adenine-flavine-dinucleotide when adenosinetriphosphate and riboflavine are added as follows: 0.2 g. of acetone kidney powder was added to the mixture of 2×10^{-6} M riboflavine, 2×10^{-6} M ATP and 8×10^{-6} M Na_2HPO_4 in 4 ml. of water, allowed to stand at 30° for 20 hours, and then heated at 80° for 5 minutes, and centrifugated. The supernatant was used as the test material (I). For control, the same mixture was heated at once and treated as above described (II). As the third set, 0.2 g. of acetone powder was added to 4 ml. of water without addition of other substances and treated in the similar manner (III). The fourth set consisted of three substances in water without addition of acetone powder and treated in the same manner (IV). The catalytic effect of the synthetised dinucleotide was tested upon the co-enzyme action for *d*-amino acid oxidase.

dl-Alanine (5 mg./ml.) was used as the substrate. 5 ml. of the apoenzyme solution ("solution B") (2) was extracted from 15 g. of pig kidney as usual.

Data from one experiment are presented by the following table.

TABLE

	I	II	III	IV
Solution B	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
Phosphate buffer (pH=8.3)	0.7	0.7	0.7	0.7
dl-Alanine	0.2	0.2	0.2	0.2
I	0.5	—	—	—
II	—	0.5	—	—
III	—	—	0.5	—
IV	—	—	—	0.5
Amounts of consumption of oxygen				
after 10 minutes (cmm.)	43	4	0	4
„ 20 „	85	7	0	8
„ 30 „	114	12	0	10

Doubtless, the results confirm our suggestion that dinucleotide is synthetised from riboflavine and ATP by the kidney enzyme.

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The Journal of Biochemistry, Vol. 37, No. 4.

ON THE CHANGE OF N-SUBSTITUTED AMINO ACIDS IN THE ANIMAL BODY. XII.

FORMATION OF KYNURENIC ACID FROM D-ABRINE IN THE ANIMAL BODY.

By

TAKASHI KOMAKI

(*From the Department of Medical Chemistry, Kyushu University, Faculty of Medicine, Fukuoka*)

(Received for publication, August 15, 1950)

The fate of L-abrine was studied by Hirohata and his co-workers (1936, 1937). They found that a relatively large amount of kynurenic acid and kynurenine were excreted in the urine of rabbits, to which L-abrine had been administered. Moreover, Chin (1938) in his laboratory demonstrated that the hematopoetic and growth promoting actions of L-abrine were as strong as L-tryptophan. Kynurenic acid can be formed by metabolic reactions from indolepyruvic acid, but to a smaller extent than from tryptophan (Ellinger and Matsuoka (1920), Berg (1934)) and its hematopoetic action is weaker than that of tryptophan (Kinami (1929), Chin (1938)).

From the reports of these investigators and their own results (1936-1938), Hirohata came to the conclusion that L-abrine must undergo demethylation to form L-tryptophan. Further, they detected, a new enzyme "demethylase", which oxidizes the methyl group to formaldehyde and removes it from abrine leaving L-tryptophan. Demethylase oxidizes only L-α-N-monomethylamino acids and does not attack the D-modifications. (Yosida and Fukuyama (1941, 1944), Yosida (1944)). Fujikawa (1938) and Gordon (1939) studied on D-abrine and found no nutritive value to the rat. However, its

intermediary metabolism in the animal body was not studied.

The present investigation was undertaken to determine the fate of D-abrine *in vivo.*

Experimental

Metabolism of D-*Abrine*

D-Abrine was prepared from racemised abrine according to Majima (1936) and Fujikawa (1938) by the cultivation of *E. coli* in nutrient medium containing DL-abrine. It melted at 279°.

$$[\alpha]_D^{20°} = \frac{-0.77 \times 100}{2 \times 0.862} - 44.66$$

Into rabbits weighing about 2 kg, which were previously fed on polished rice for 5 days, 0.5 or 0.25 g. D-abrine dissolved in $NaHCO_3$ or Na_2CO_3 solution was injected subcutaneously twice a day for 3 days. Their urine were collected for 48 hours after the last injection. To the urine was added H_2SO_4 to a concentration of 5 per cent. It was overlayed with ether, and kept in the ice-box for 24 hours. The precipitated crystals (crude kynurenic acid A) were centrifuged out. To the supernatant liquid was added 10 per cent $HgSO_4$ solution, and the mixture was filtered after 24 hours. The mercury was removed from the residue as HgS and the filtrate evaporated *in vacuo.* The crystalline substance, precipitated during next 24 hours in the ice-box, was centrifuged (crude kynurenic acid B). A and B were combined, dissolved in ammonia water, filtered, precipitated by acidifying with HCl, and centrifuged. The residue was recrystallized from water. (Table I)

The mother liquor of B was evaporated under diminished pressure removing SO_4 gradually with baryta, however no sulfate of methylkynurenine or kynurenine could be obtained in spite of every effort.

Finally the SO_4 was removed with baryta quantitatively and distilled *in vacuo.* The unchanged D-abrine crystallized and this was filtered and recrystallized from water. m. p., 288°.

TABLE I

Exp. No.	Rabbit No.	Body wt. (g.)	D-Abrine administered (g.)	Kynurenic acid Weight (g.)	Kynurenic acid per cent	Unchanged D-abrine Weight (g.)	Unchanged D-abrine per cent
I	1	1850	3	0.963	21.4	1.523	33.8
	2	2100	1.5				
II	3	2800	3	1.1941	13.3	3.1696	35.2
	4	1900	3				
	5	2100	3				

The mother liquor of unchanged abrine was evaporated in vacuo, whereupon a red oily substance containing several milligrams of prismatic crystals was obtained. This was spread on a porous plate and kept in a desiccator, but it turned dark brown slowly and clean crystals could not be obtained. To our regret, thus, we could not examine it well, but there was no doubt that it was related to the kynurenine yellow reported by Kotake (1931).

Analyses :

Kynurenic acid $C_{10}H_7NO_3$	calculated			N 7.41	
	found			„ 7.55, 7.26, 7.45, 7.37	
D-Abrine $C_{12}H_{11}N_2O_2$	calculated	C 66.03	H 6.47	N 12.84	
	found	„ 66.36	„ 6.05	„ 12.35	
		„ 66.47	„ 6.19	„ 12.30	

Hematopoetic Action of D-Abrine

Five rabbits were fed on the same food 3 weeks before the beginning of the experiment until the end of it, and made anemic with phenylhydrazine. Under the administration of D-abrine to two of them, the restoration of the red blood cells and the quantity of hemoglobin were determined. The results are given in Table II.

TABLE II

Rabbit No.	Body wt. (g.)	D-Abrine (mg)	Restoration of Hemoglobin (days)	Restoration of Red blood cells (days)
1	2020	21.6	18	18
2	1970	21.6	18	20
3	2490	0	21	21
4	2540	0	19	19
5	2270	0	23	22

As it is seen on Table II, hematopoetic effect of D-abrine is far less than that of L-abrine studied by Chin, and more comparable to that of indolepyruvic acid.

DISCUSSION

The tryptophan-pyrrolase described by Kotake seems capable of opening the pyrrol ring of indole in the tryptophan molecule, despite of the configuration of α-carbon atom of the side chain, since Y. Kotake Jr. (1937) obtained D-kynurenine by the administration of D-tryptophan *per os* to rabbits. If the methyl substituted α-N does not interfere with the action of the enzyme, it is very probable that one will obtain D-α-N-methylkynurenine by administration of D-abrine. Contrary to our expectation, however, a relatively large amount of kynurenic acid and a small quantity of kynurenine yellow-like substance were obtained, and neither methyl kynurenine nor kynurenine was isolated. It is very remarkable that rather a large amount of kynurenic acid was obtained without kynurenine. By the parenteral administration of D-tryptophan, no definite substances were isolated by Kotake Jr. (1936).

According to Keilin and Hartree (1936), and Handler *et al.* (1941), D-amino acid oxidase can remove methylamino group from the DL-α-N-methylamino acid leaving α-ketonic acid. Kenmochi in

our laboratory, however, could not confirm this action of the enzyme on D-abrine. Since demethylase can not act on the D-modification as above mentioned, the metabolic course of D-abrine to kynurenic acid is not still clear and needs further investigation. Nevertheless, it seems probable that an unknown enzyme acts on D-abrine to produce indolepyruvic acid, which will turn into kynurenine yellow and kynurenic acid subsequently.

Summary

1. A relatively large amount of kynurenic acid together with a small quantity of kynurenine yellow-like substance were obtained in the urine of the rabbits, to which D-abrine was administered.

2. About 1/3 of administered D-abrine was excreted in the urine unchanged.

3. The hematopoetic action of D-abrine is very weak.

4. The course of the formation of kynurenic acid from D-abrine has been discussed.

We are indebted to the Department of Education for the grant of "Research Fund for Science", with the aid of which, this work was carried out. (Ryozo Hirohata)

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Errata

On the Quantitative Interpretation of Electrophoresie Experiments with Tiselius Apparatus.

By K. Shimao.

Vol. 37, No. 2 p. 229 Table V (Continued)

1. Apparent %, second line

mistaken	correct
57.5 8.7 11.4 6.8 18.6	58.0 8.4 10.1 6.4 17.2

2. True %, second line

mistaken	correct
52.3 11.1 .2.3 8.9 19.4	52.8 11.1 11.2 7.2 18.0

INDEX TO AUTHORS

INDEX TO SUBJECTS

ABSTRACT

from

The Journal of Japanese Biochemical Society.

(Vol. 18, 1944.) p. I—X.

1. Studies on the so-called Combined Form of Vitamin C in Animal and Vegetable Tissues, I. On the Existence of so-called Combined Form of Vitamin C in Animal and Vegetable Tissues. Tadashi Nagayama. p. 1-13.

Results of acid hydrolysis of pure ascorbic acid soln. and also tissues at 90° for 90 min. and at 100° for 30 min. were compared. Little difference was thus observed between the two methods provided that the hydrolysis was carried out in CO_2.

On hydrolysis of both animal and vegetable tissues "indo-phenol reducing. value" was increased with the increase of acid concn. Most of the reducing substance detd. were oxidised by ascorbic acid oxidase (I). Free, vitamin C (II) on the contrary decreases on acid hydrolysis.

With tissues contg. little II the combined form could be safely detd. but with those contg. much III the detn. of I sometimes failed due to the predominant decrease in III.

When a ppt. obtained by the addn. of a protein precipitant was hydrolyzed after having been washed completely with metaphosphoric acid soln. the II was produced, which reduces "indophenol" value and was specifically oxidised by I. II present in both animal and vegetable tissues mostly seemed to produce II upon the acid hydrolysis.

2. Ibid. II. A Vitamin C-like Reducing Substance Produced by the Hydrolysis of Carbohydrates. Tadashi Nagayama. p. 14-25.

Fructose and many other carbohydrates produce on hydrolysis a vitamin C-like substance (I) which was oxidised specifically by ascorbic acid (III) oxidase (II). The substance produced from fructose and from both animal and vegetable tissues, however, was not least influenced by oxygen in 4N. HCl soln. at 90° within 90 minutes. When II was subjected to acid hydrolysis (4N.HCl, 90°, 90 min.) in the presence of fructose and in oxygen atm. 60 to 70 % of it could be recovered. This high recovery was due to the protective action of I formed by the hydrolysis of the fructose. Animal and vegetable tissues treated in the same manner did not show the protective action like fructose, nevertheless when III was added to the hydrolysate of the tissues the protection action (90 %) recovery was observed, which was stronger than that of fructose.

I produced from fructose and from the tissues, were alike in various respects. They had in turn similarity to III with the exception that the formers were strongly resistant to the oxidation by oxygen.

3. Biochemical Studies on Chaulmoogra Oil. Decomposition of Chaulmoogra Oil. Shokichi

Koyanagi. p. 26-28. cf., J. Biochem. 32, 317 (1940); 35, 61 (1942); 31, 109 (1940).

The method employed for this study was reported in preceeding report as quoted above. In the present expt., it was shown that chaulmoogra oil was decomposed by tissues of liver and kidney. It was more rapidly decompd. than olive oil.

4. On Creatine-mutase. Yoichi Kanai. p. 29-40.

Gram-positive rod-shaped bacteria isolated from soil. The bacterial suspension which has been heated for 30 min. at 55° was no more capable of creatinine decompn., but contains a new enzyme which catalyzes reversible transformation of creatinine and creatine. The optimal pH of the enzyme action was 7. The equilibrium point was the same no matter whether it was reached from creatinine or from creatine. Moreover, the value of the equil. const. was the same with or without the enzyme. The change of the equil. const. was temp., followed the van't Hoff isochore.

5. Studies on the Carbonydrase VI. On the Difference between Glucosidase and Galactosidase. Koichi Kobayashi. p. 41-57.

Four enzymes, β glucosidase and β-galactosidase of both Takadiastase- and emulsin-types, present in nature in isolated form were used to hydrolyse phenol-, o-cresol-, m-cresol-, and p-cresol- β-glucosides. Thus, the order of hydrolysability due to the nature of aglucone was detd. by measuring velocities of hydrolysis, as follows:

Emulsin-type β-glucosidase (Proteus vulgaris X_{19}, 0-type)
o-cresol- ≫ m-cresol- > phenol- ≧ p-cresol-β-glucoside

emulsin-type β-galactosidase (Bac. coli communis)
o-cresol- ≫ m-cresol- > phenol- ≧ p-cresol-β-galactoside

Taka-type β-glucosidase (indian millet)
phenol- > p-cresol- = m-cresol- ≫ o-cresol-β-glucoside

Taka-type β-galactosidase (hog spleen)
m-Cresol- = p-Cresol- > o-Cresol- > phenol-β-glucoside

As indicated above, with the two emulsin-type enzymes, the orders of of hydrolysability of β-glucosides and β-galactosides were the same, corresponding to that for β-hexosides by emulsin. With the two Taka-type enzymes, the orders for β-glucosides and β-galactosides differed from each other, nevertheless the each order corresponded to that of hydrolysis by Taka-diastase.

p-Nitrophenol-β-glucoside is hydrolysed by emulsin much faster than o-cresol-β-glucoside, but by Taka-diastase hydrolysed slightly faster than o-cresol-β-glucoside and slower than the other three glucosides. Hydrolysis of p-nitrophenol-β-galctoside by emulsin was faster than that of o-cresol-β-galactoside, but its hydrolysis rate by Taka-diastase was about the same as m- and p-cresol-β-galactoside.

Dialysed rabbit liver autolysate contains both enzymes of Taka-type and Emulsin-type β-glucosidase and β-galactosidase. Dialysed ox liver autolysate contains Taka-type β-glucosidase in addn. to both enzymes of Emulsin-

type, and in dialysed hog liver autolysate the both enzymes of Emulsin-type have been found but not enzymes of Take-type.

6. Determination of Specific Gravity of Serum by Falling Drop Method of Minute Amount. By Haruhisa Yoshikawa. p. 58–62.

The Barbour and Hamilton method was slightly modified, namely one drop (0.01 ml.) of test liquid was dropped in a mixt. of xylene and chlorobenzene, and sp. gr. was calcd. from its falling velocity. Thus, the sp. gr. (20°/20°) of human serum was found to be 1.0284.

7. Studies on Aneurinase: I Aneurinase, A New Enzyme, and its Nature. By Akiji Fujita & Isamu Numata. p. 63–70 cf., J. Biochem. **35**, 89, 419 (1942).

Thiamine (I) added to various animal and vegetable tissue exts. was not recovered after some hrs. This led to the discovery of a I-decompg. enzyme present in many shell-fishes, named the enzyme *aneurinase* (II).

The nature of II present in Tapes philippinarum (III) was as follows: (1) 2 ml., of 10-fold dil. ext. of III completely decompd. at least 0.5 γ of I ordinarily within 60 min. at 37°; (2) II was not dialysable.; (3) By heating, 15 min., 80° most, 15 min., 90° whole., and 60 min., 75° about 2/3 of its activity was lost.; (4) The optimal pH of its action was approx. 5.5 and below pH 3 it exerted no action. At pH 9 it was fairly active and at pH 10 more than one half of its activity at pH 5 is observed. The optimal temp. of its action is 60° and at 37° about one half of its activity at 60° is seen. Presence of O_2 did not influence its activity. II was comparatively unstable, e.g., its soln. losed completely its activity when stored in an ice-box for 5-6 days, but its soln. satd. with $(NH_4)_2SO_4$ losed to no noticable extent under the same conditions.; (5) II was found even in putrified tissues of III.

Adjustment of pH of the ext. soln. to ca. 4.5 caused pptn. of large part of protein but not II. The conditions can advantageousely be employed for the prepn. of the II.

8. Studies on the Transition of Physiological Conditions of the New-born, X. Transition with Growth of Plasma Protein Fractions and of Blood Lipoids (Total Cholesterol and Lecithin) By Keiso Rin p. 77–86, cf., J. Biochem. **31**, 205 (1940).

Using new-born puppies proteins and lipoids in blood were detd. daily. Total plasma protein and its fractions were detd. by Howe's method cholesterol by the colorimetric method, and lecithin from the value of phosphoric acid colorimetrically measured.

The conclusions wege as follows: (1) Total plasma was increased rapidly for a week, and then gradually. 1st day, 3.35 g./dl; 7th day, 4.522 g./dl; 60th day, 5.431 g./dl; at maturity 6.19 g./dl. (2) The plasma protein fractions, (a) Albumin was very slowly increased with growth (1st day, 2.404 g./dl; 60th day, 2.994 g./dl; at maturity, 3.449 g./dl.) (b) Total globulin increases rapidly for 1 week after birth: 1st day 0.999 g./dl; at maturity 2.304 g./dl. (c) Euglobulin concn. was very low for 1 or 2 days, but it increases rapidly from

the 3rd to 7th day, and then gradually. After the 21st day it was increased little: 1st day, 0.04 g./dl; 7th day, 0.237 g/dl; 21 st day, 0.317 g./dl. (d) Pseudo-globulin I was also low at birth but it was increases rapidly from 3rd day, to 14th day, and then slowly: at birth, 0.591 g./dl; 14th day 1.025g./dl; at maturity, 1.267 g./dl. (e) Pseudo-globulin II was inereased gradually untill maturity: 1st day, 0.375 g./dl; at maturity, 0.612 g /dl. (f) The transition of fibrinogen concn. with growth is similar to that of pseudo-globulin: 1st day, 0.148 g./dl; 14th day, g./dl; On the 30th day the value for the matured was attained. (g) The A/G ratio was the highest for the first two days but decreases rapidly until the 7th day, after which the decrease was slow. (3) Blood cholesterol concn. was low (0.1236 g./dl) for the first two days and after the 3rd day it was rapidly increases reaching to the max. on the 14th day. After this it was decreases again (0.1541 g./dl on the 60th day) until finally the value for the matured. (4) The transition of lecithin concn. is similar to that of cholesterol, i. e., 0.1442 g./dl on the 1st day, 0.3038 g./dl on the 14th day, and afterward the value decreases. (5) The change in residual N value with growth was the same as those reported in the previous paper. The ratio of blood cholesterol to lecithin was slightly high at first and decreases gradually afterwards, but through all gtages of growth, the ratio was considered to be fairly const.

9. Ibid. XI., Transition with Growth of Distribution of Chemical Components, especially Phospholipids, of Cerebrum of a New-born Puppy. By Keiso Rin p. 87–96.

Using new-born puppies the change with growth in weight, volume, sp. gr., water content and dry residue of the cerebrum and the ratio of cerebrum wt. to body wt. were detd. in addn. to the detn. of its chemical constituents, such as cephalin, sphingomyelin, lecithin, cholesterol, total N and ash. The results thus obtained was as follows: (A) Physical measurements.: (1) The change of cerebrum wt. with growth was conspicuous. 1st. 2nday, 6.9) 7.00 g. the 7th, day, 12.5) g. the 30th day, 29.94 g.; the 60th day, 48.65 g. at maturity, 58.10 g. (2) The increase of volume was in parallel with the wt. increase. 1st day, 6.70 ml. 7th day 12.08 ml. 30th day, 28.88 ml.; 60th day, 46.92 ml, at maturity, 56.04 ml, (3) The change in sp. gr. was very slight. 1st day, 1.030; at matunty. 1.037 (4) The ratio of cerebrum wt. to body wt. was high at the initial stage followed by rapid decrease: 1st day, 0.0300; 7th day, 0.0207; 30th day, 0.0173; 60th day 0.0123; at maturity, 0.0035.

(B) Chemical Detn.: After the physical measurements, the cerebrum was cut and ground up to a homogeneous paste. With a portion of the paste water content, dry residue, ash, and total N were detd. In accordance with the Thannhauser method, the remaining portion was frozen by dry-ice and then dried in a decicator over P_2O_5 under a reduced pressure. Lipoid was detd. with the dried material by the Thannhauser method Phospholipids was detd. by the Erickson method and cholesterol by the method described in the previous report (1) The change

in water content with growth was most conspicuous: 1st day 89.53 %; 3rd day, 88.74 %, and such high value was kept until the 14th day, after which the decrease became conspicuous. 30th day, 85.45 %; 60th day 82.39 %; at maturity 79.96 %. (2) The change in the dry residue with growth was just the reverse of the water content. (3) (a) With the fresh tissue the total N was comparatively small at the initial stage, increasing gradually with growth. 1st day, 1.03 %; 7th day, 1.11 %; 30th day, 1.30 %; at maturity 1.66 %. (8) With the dried tissue the total N was high at the initial stage but decreases afterwards. 1st day, 9.81 %; 7th day, 9.35 %; 30th day, 9.22 %; 60th day, 8.32 %; and at maturity 8.20 %. The fact probably indicated that in cerebrum the increase with growth of deposited nitrogenous substance, say proteins and nitrogenous lipoids was less than that of non-N substances. (4) Ash content % which was markedly increased with growth until the 7th day 1st day 7.83 %; 7th day, 10.16 %; 7th day, 10.86 %, corresponding to the value at maturity, (5) The total lipoids content was the lowest on the 1st day (11.19 %); 2nd day, 12.19 %; 3rd day, (the increase is fairly rapid,) 7th day, 14.66 %; 30th day 19.50 %; and 60th day 24.44 % (approx. the value at maturity).

The substances participating the transtion of total lipoids content were mainly cephalin, choilesterol, lecithin, sphingompelin. (a) Cephalin, the principal constituent of cerebrum lipoids, changed in parallel with that of the total lipoids. 1st day, 6.25 %; 7th day, 8.51 %; 30th day, 11.55 %; 60th day, 12.73 %; at maturity, 14.61 %.

When expressed as percentages with regard to the total phospholipids the cephalin contents were as follows: 1st day, 55.85 %; 7th day, 58.05 %; 30th day, 59.23 %; at maturity, 59.78 %. The cephalin occupies more than one half of the total lipoids content. (b) The transition of sphingomyelin content with growth kept pace with that of cephalin. 1st day, 3.09 %; increasing gradually with growth although not so distinctly as in the cases of cephalin and lecithin. Expressing as % for total phospholipids sphingomyelin was highest at the initial stage of grow h and then decreases 1st day, 27.61 %; 7th day, 23.81 %; 30th day, 22.46 %; at maturity 21.24 %. (c) The lecithin content at the initial stage was considerably low but but it was increased markedly with growth. 1st day, 1.85 %, 7th day, 2.66 %; 30th day, 3.57 %; 60th day, 3.96 %; at maturity, Expressing as % to total phopsholipids 1st day, %; 7th day, 18.14 %; and afterward increased but not so distinctly as cephalin. (d) The chang of total choline-phospholipid with growth was as follows; 1st day, 4.94 %; 7th day, 6.15 %; 30th day, 7.95 %; 60th day, 8.66 %; at maturity 9.83 %.

The main snbstance constituting the total choline-phospholipid seemed to be lecithin. Contrary to cephalin the percentage of choline-phospholipids to total phospholipids, howerer, gradually was decreased with growth: 1st day, 44.15 %; 7th day, 41.95 %; 30th day 40.77 %; at maturity, 40.22 %. (e) The transition of cholesterol content with growth was as a whole the same as that of cephalin. It increased gradually

until the 3rd day. Afterwards the increase became fairly rapid: 1st day, 3.22 %; 7th day, 5.29 %; 30th day 9.18 %; 60th day, 10.79 %; at maturity. 12.35 %.

10. On the Formation of Carbohydrates from Fat in Animal Body, III. On the Respiratory Quotient and D: N Ratio of Urine under the Influence of Fat Diet and Phlorizin Injection. Shigeo Nakamura and Katsumi Ando p. 97–99. cf. J. Biochem. 35, 1, 9 (1941).

Using pigeons the formation of carbohydrate (I) from fat (II) was studied through the measurement of the respiratory qnotient, R. Q. or of D/N ratio in case of phlorizin diabetes.

R. Q. measured with pigeons fed on II contg. no I but protein (III) in physiol. min. amt. was found less than and sometimes more than 0.7. This disagreement was probably due to the oxidation of I immediately after its formation from II.

R. Q. detd. after the injection of phlorizin (IV) (1 ml., 2 %, in Na_2CO_3 sol. per 100 g. body wt.) with pigeons fed on II were av. 0.681 in 1 hr. and 0.614 in 3 hrs.

With pigeons fed on II, R. Q. was av. 0.606 in 1 hr. and 0.614 in 3 hrs. after the injection of IV, while before the injection of IV, R. Q. was av. 0.681. In every case, R. Q. was decreased markedly after the injection. This finding supported the formation of I from II.

D: N ratio detd. with urines of IV-injected pigeons was in av. 3.84 while before IV. injection, it was 0.129, thus indicating the marked increase in the ratio.

On the contrary IV excretion showed little change in this case and therefore the marked increase in the ratio indicated the marked increase in amt. of sugar.

This in turn indicated that the sugar excreted was not produced from III but from II.

11. Ibjd. IV., Effect of Adrenaline, Insulin, and Phlorizin upon the Carbohydrate Formation from Fat. Masatoshi Hara p. 100–106 (1944).

Two 2 groups of pigeon, were fed: the one with normal diet and the other with II, plus adrenaline (V,) insulin (VI), or IV-injection, and their effect upon the metabolism of II and I in liver was investigated with the purpose of elucidating their effect upon the I formation from II. The results were as follows: (1) V-injection bringed about little difference in the amts. of II and glycogen (VII) in liver of a pigeon fed on normal diet. It is the well-known fact that V mobilizes VII in liver to bring about the increase of blood sugar and glucosuria. The reason why the VII in liver was not decreased by V in the presen expt. was that I in the diet constantly compensated its decrease. (2) The content of II in liver of a pigeon fed on II for 10 days was av. 8.21 % after the administration of V for 5 days, while that of the control (fed on II, without V) was 5.00 %. VII in liver was av. 1.12 %, almost same as that of control (0.99 %). (3) In the previous paper the body of a pigeon fed on II, I was formed from II. When V was injected

to a pigeon under such conditions, VII in liver still remained approx. the same as that of the control. This fact may be considered to indicate that the decrease in I due to the V-injection, is compensated by the sugar production from II. Formation of I from II, should go on much vigorously in the pigeon having received V-injection than the control fed on II. II in liver of the pigeon with which V-injection was greater than that of the control. (4) II and VII in liver of a pigeon fed on normal diet for 10 days and afterwards given the VI-injection for 5 days were are practically the same as those of the control. This may be understood by the explanation that the possible decrease in VII in liver due to the action of VI is supplemented by I of the diet. (5) II in liver after VI, injections of a pigeon fed on II was av. 7.47 % while that of the control was 5.00 %. VII in liver of the former was 1.13 % and that of the latter was 1.12 %. (6) After a careful expt., Yoshida (personal communication) concluded that VII in liver glycogen was decreased by VI-injection. A pigeon fed on II showed, after repeated injection of VI, practically no difference in VII content from the control. This was evidently due to the supplement of VII decrease by sugar formed from II. This relation was similar to the case of the V-injection. (7) Even after IV-injection, II and VII in of a pigeon fed on normal diet stayed practically thes ame as those of a control pigeon on normal diet. (8) After IV-injection to a pigeon fed on II, VII in liver stays practically the same as the control fed on II, but II in liver of the former was 7.01 % againat 5.00 % of the latter, thus indicating marked increase in the former. (9) These finding may be interpreted as follows: Within the body of a pigeon fed on II, I formation from II is already going on. If IV is injected to such an organism, I is decreased and in order to compensate this decreases of I, I-production from II becomes more vigorous. As a result, II is transfered to liver, thus increasing II in liver.

Therefore, it was concluded that IV also accelerated the I formation from II.

12. Supplement to the Stereochemistry of Steroids. On the Steric Arrangement of H at C_9-Position. Kazumi Yamazaki. p. 107–114.

Hückel *et al.* and Vavon *et al.* reported that the dehydration from the secondary OH-group and from the adjacent tertiary H takes place far more easily when the both groups are in trans-position than in cis-position.

The author presumed that this rule was valid for the dehydration of cis-trans isomers like chenodesoxycholic acid (I) and ursodesoxycholic acid (II), and studied, under various conditions, the dehydration in which C_7 OH-group participated. The presumption was correct.

The fractionation of unchanged dioxycholanic acid and dehydrated product was carried out according to the Wieland, Seibert, and Heki method; the ether soln. of the reaction mixt. was extd. with 25 % HCl, taking the extd. part as the unchanged and the part remaining in ether layer as the dehydrated product (oxycholanic acid).

Although the fractionation method Wieland *et al* was originally applied to satrd. oxycholanic acid, the present *expt.* indicated its applicability to unsatrd. cholic acid.

Since the dehydration took place far more easily in I acid than in II desoxycholic acid, it was considered that it under went trans- in I and cis-transformation in II. Hence C_7 OH-group and C_8-tertiary H of I were transoriented and those of II cis-oriented.

In the case of I, C_7-OH-group and C_{10}-Me-group were mutually in trans-position. B-ring and C-ring were mutually trans-oriented. Hence, with regard to C_{10}-Me group C_8-H were in cis-position and C_9-H in trans-position.

It is of interest to note that the steric arrangement of the C_9-H assumed by Ruzika without any experimental background corresponds to the present author's conclusion.

13. On the Change of N-Substituted Amino Acids in the Body. X. Destiny of N-Dimethylphenylalanine in the Body. Senden Fukuyama p. 115-118; c. f. J. Biochom. 34, 429 (1941).

Urine of rabbits which received injection of N-dimethylphenylalanine (I) was examd. for hippuric acid, phenaceturic acid, substances capable of combining with bisulfite, and mehtylamine. No increase in their amounts were detected. Phenylpyruvic acid was not isolated, while approx. 70 % of I was recovered.

In view of these findings it was considered that I did not undergo any conversion *in vivo* as was already pointed out by Knoop (1927). This supports the dehydrogenation theory proposed by Wieland.

14. Distribution of Vitamin B_2 in Animal and Vegetable Kingdoms, I. Sasuke Okubo p. 119- 138.

By use of Okubo & Fujita method, contents of vitamin B_2 in animal and vegetable tissues were detd. And the decreases of the vitamin B_2 content in foods during processing were studied.

The detn. extended to more than 200 kinds of vegetable foods, including leaves, snems, roots, rhizomes, tubes, bulbs, corns, fruits, seeds, algae, fungi, and their processed products; and nearly 200 animal foods, including milk, eggs, and molluscae, crustaceae, fish, and animal meats in addn. to their processed foods,

15. Distrubution of Vitamin B_1 in Animal and Vegetable Kingdoms IV. Keisaburo Dohi p. 139-150 c. f., Ibid., 35, 89, 419, 431 (1942).

As the continuation of the work reported (references above indicated) vitamin B_1 content of nearly 300 foods both animal and vegetable origins are reported.

16. Biochemical Studies on Phospho-lipids, I. On Acetal-phosphatid (Plasmalogen). Kimiyoshi Ohno p. 151-158.

Only the three phospho-lipids; lecithin (I), cephaline (II), and sphingomyelin (III), have so far been isolated and their existence in animal bodies is definitely proved. The actions in the living body of these three phospho-lipids, however, may be considered not necessarily the same. It is, therefore, important to obtain the information

with regard to their biochemical significance by detg. their distribution in various tissues with methods which detn. these phospholipids separatory from each other.

With the purpose of detg. the three known phospholipids; I, II, and III separatory from each other bull's brain, were extd. in succession with aceton, alcohol, ether, and hot alcohol to obtain various lipoid fractions. Plasmologen (IV) was found to be contained especially in phospho-lipid fractions and unable to be separated from I and II by mere separatory method based upon the solubility differences.

Thiosemicarbazone of plasmal was separated in crystl. form from I, II and IV obtained from alc. fraction by aceton-pptn. and purification. The results of analysis and its m. p. (90–100°) indicated that it was a mixt. of stearal and palmital, the former being contained in somewhat greater percentage. II obtained from the ether-fraction by purification and re-pptn. with alcohol gave distinct plasmal reaction.

After heating II with N/10 HCl at 37~40° for 4 hrs., no plasmal reaction for IV was shown.

A portion of plasmal obtained from IV in the above process was converted into crystl. thiosemicarbazone, which was considered from m. p. (96–97°) to be a mixt. of roughly equal parts of stearal and palmital.

17. Ibid. II. On the Presence of Amino Acid in Cephaline Molecule. Kimiyoshi Ohno p. 159–166.

The author succeeded in separating serine and cholamine as their naphthaline-sulfonyl derivatives from the hydrolysate of II free from IV.

From this finding, he concluded that II was a mixt. of cholamine-cephaline (V) and serinecephaline (VI), and VI was the precursor of V. Inconsistencies observed in the properties of cephaline was thus explained.

The author proposed the hypothesis that in an organism V produces through decarboxylation of VI which in turn gives rise to I.

18. Experimental Study on the Reduction of Dehydroascorbic Acid in Organs. Tohru Kinkawa p. 167–182.

To explain the reduction of dehydroascorbic acid (I) in organs there is proposed either enzyme theory or thiol group theory. The author carried out expts. from various angles to investigate the validity of the two theories and brought out the following findings:

(1) Under anaerobic conditions (particularly in N_2 gas) I was reduced in phosphate buffer.

(2) Hormones of both adrenal cortex and medulla did not reduce I.

(3) An enzyme did not participate in the reduction of I in organs.

(4) Although a part of I was reduced by glutathion and other thiol compd., most part of the acid seemed to be reduced by other compds. than by the thiol.

(5) The substance participating in the reduction of I in organs possessed the following properties: (a) The organs contg. a substance reducing I were in the order of its content, adrenal gland, spleen, small intestine, liver, and kidney. (b) I-reducing substance contained in the supernatant

liquid of tissue brei was not destroyed by heating but its reducing power was rather increased. (c) The reducing power for I cf deproteinated adrenal tissue fluid was suppressed by the addn. of ascorbic acid and was also somewhat decreased by heating. (d) The reducing substance was insoluble in ether. (e) It was not ppted. by acetone. (f) It was dialyzable.

19. Studies on Metabolism of Cholic Acid, VII. Conversion of Dehydrocholic Acid by Organs in Vitro (Supplement I). Yasuo Kihara p. 183–186.

This study was carried out to supplement the results obtained by Saha (1940), since Saha's expts. need reinvestigation as there was still discussion about the technique.

Here the exptl. results with muscle was compared. with those with liver and small-intestine and the extent of reaction due to the difference in substrate concn. and amt. of tissues employed were considered in view of the diagramms with dehydrocholic acid (I) concn. and time.

I was detd. by the m-dinitrobenzene method. The conclusion was as follows: (1) Muscle brought about the change of I in such a way as to decrease the value of carbonyl group at 3-position. This ffect was somewhat smaller than those of liver and smoll intestine. (2) The higher was the substrate concn. the higher was the velocity cf this reaction. Beyond a certain substrate concn., the difference in velocity seemed to disappear. (3) The greater the amt. of tissue added, the greater was the velocity at the initial stage. Amt. of I reacted with muscle and small intestine at final stage became the same, while it markedly deffered by using different amt. of liver tissues.

This indicated that there was a qualit. difference in the reaction mechanism between the reaction with liver and with muscle or small intestine.

20. Ibid., VIII. Conversion of Dehydrocholic Acid by Organs in Vitro (Supplement 2). Yasuo Kihara p. 187–190.

The expt. was carried out with the purpose of elucidating the nature of the change of I. The author considered that the apparent qualit. difference between the effects of liver and small intestine or muscule was due to the difference in affinities of these tissues with I and the reaction in which the decrease of carbonyl group occurred by the addn. of these tissues was considered to be of the same nature.

As the result of the trial to obtain a reaction product formed by the action of I with rabbit muscle tissue, the plate-formed substance of m. p. 216, having acid reaction was obtained. Its chemical properties, was not detd. on account of its too small yield.

ABSTRACT

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21. Studies on tyrosinase in the growing paddies. Shigeru Komatsu, Kinkichi Sato, & Ayako Ito. 191-197.

The water exts. (pH 7.4) of leaves, stems, roots or seeds of paddy were used as the enzyme for tyrosinase activity detns. These expts. were carried out at 6 periods of the growth. O_2 uptake by the enzyme soln. with tyrosine in leaves, stems, or roots, was at max. value in the beginning and gradually decreased; in seeds, it was, max, just after coupling.

Insoculation of *Pricularia oryzae*, caused as twice O_2 uptake as the normal. These observations were discussed in relation to *pricularíosis* of paddy, the leaves of which showing yellow-brown dotts, that seemed to be melanin formation.

22. The change of cholestenon and cholestanon by mouse liver tissues. Yasuw Kihara, 198-202.

Each 2 ml. of cholestenon (I) or cholestanon (II) emulsion (0.8g/dl. of aceton) was incubated with 1 g. of fresh mouse liver slice at pH 7.1, 38°, for 60 min. The remaining amts. of I or II was detd. as "$C_{(3)}$-carbonyl value" by m-dinitrobenzol reaction. The reduced amt. of I was less than that of II in the medium soln. as well as in the whole mixt. by which the amt. of I or II absorbed to the slice were cacld. as follows: the absorption of II was much more than that of I at initial period, particularly by 10 min., and approaching to the same max. value after 60 min. of incubation. This difference between I and II, secmed to be due to the properties of unsatrd. α, β- keton.

23. Biochemical studies on guanidine body IV. The estimation of glycocyamine in urine. Shinichi Shibuya, 203-206.

To 1 ml. of 4 times diluted urine, add 5 ml. of 0.1 N H_2SO_4, 1 g. of Japanese acid cray, wash the ppt. 4 times with 5 ml. of 0.1 N H_2SO_4. To the ppt. thus obtained, add 1 ml. of 10% EtOH, and 2 ml. of 10% Na_2CO_3, filter through a glass-filter. To this filtrate, add 0.5 g. of Na,K-tartarate, and 20 ml. of 10% NaOH, make 10 ml. with dist. water. The standard soln, contd. 1 ml. of 1 mg. % glycocyamine, To both, add α-naphthol and hypobromite to develop the color of Sakaguchi's reaction, according to Weber's method (J.B.C. 109, XCVI, 1935). To eliminate the interfering. Creatinine and urea in the normal concn. of urine, the 4 times-dilution was found to be preferrable after the various recovery tests.

24. Quantitative determination of sucrose in urine. Yoshi Maehata, 207-213.

To 10 ml. of soln., contg. 1 ml. of urine and 2 ml. of 0.1 N H_2SO_4, add

Lloyd reag., mix for 3–4 min., centrifuge; (1) To 0.5 ml. of the supernatant (I), add 7.5 ml. of H_2SO_4 (6:4). 0.25 ml. of 2% orcin-H_2SO_4 soln., heat at 100° in a bath for 7 min, cool, and run the colorimetay, which may give the total sugar (α). (2) To 2.0 ml. of I, add 1 ml. of 30 g./dl. NaOH, heat at 100° in a bath for 15 min.— by this procedure, gllucose is destroyed— make pH 6.0, and add dist. water to the 4.0 ml. mark. To this hydrolysate, add 0.5 ml. of orcin reag., and run colorimetry, which may give the hydrolizable sugar (β). The glucose amt. is given by $\gamma=\alpha-\beta$. This method was compared with the fermentation method by yeast, and applicated to urine and blood of rabbits or human beings administered with sucrose.

25. The decomposition of cephalin by Takadiastase. Toshio Yoshinaga, 214–215. 0.5% colloidal cephalin soln. which had been already made by mixing with buffer soln., was incubated with Takadiastase. Cephalin was ppted. with 10% Fe-citrate soln., and amino-N in the supernatant was detd, as the enzyme activity, with Van Slyke's method. The optimum pH was found to be pH 4.0.

26. Proteinase contained in Takediastase preparations. Teiji Ashida, 309–324. 50 g. of Takadiastase (Sankyo Co., Ltd.) was dissolved in 200 ml. of water, discard off the ppt. formed by MeOH (finally 50 %). ppt. by MeOH (finally 70 %), was dried and keep the brownish-yellow powder, which contd. 0.095 mg of Fe/g.

This powder was dissolved in water (10 %), transferred into a collodium sack, dialyze against twice vol. of aq. dist. water for 3 days. The outside liquid with dark-brown color was used as the enzyme soln (I). I acted on edestin, casein, egg-albumin, gelatin, and boiled serum. where the acceleration by KCN, H_2S, or ascorbic acid was very little. I did not act on dipeptide. Lower amylolytic activity of I was observed, while higher amts. of amino-N was found in I.

27. Studies on aneurinase II. Akiji Fujita, & Isamu Numata, 325–335.

Aneusinase (I) of shell fish is inactivated in acid soln. (pH 1). After the action of I on vitamin B_1 (II), color reaction of II disappears. The attack of I on II is not at the NH_2-group of pyrimidin, but rather the bond between pyrimidine and thiazole groups. It is concluded that, I is consiting of both thermostable coenzyme and thermolabile apoenzyme, the latter being absorbed to burned adsol at pH 5.5. Ppt. of I with $(NH_4)_2SO_4$ (1.0 satrn.) restores its activity by the addn. of II.

28. Conversion of N-substituted amino acids *in vivo*. XI. Fate of phenylalaninebetaine. Senden Fukuyama, 336–338.

Phenylalaninebetaine (I) was injected to rabbits or dogs, and urines were analyzed: no increase of Me-amine, hippuric acid, or B.B.S. (bisulfitebinding substance) was observed. The recovery of I was about 70 %.

29. Studies on aneurinase III. The distribution of aneurinase. Akiji Fujita, & Isamu Numata, 339–346.

The activities of aneurinase (I) of more than 100 species of animals and plants were measured by detg. the

vitamin B_1 decomposition. The following distributions of I were observed: none of I, in mammals, fishes, fungi, and higher plants; very few of I, in insects, and lower animal species; only in liver of crustacea; much contained and widely distributed, in shell fishes, particulary in liver; distributed widely in most of bacteria.

30. The influence of fat-diet on the paralysis by diphtheria toxin. Tsuneji Shoji, 347–358.

Chickens, 6 days after birth, were fed with daily 5 g. of exptl. diets for 10 days. Diphtheria toxin (I) (1/30 D.l.m.) was injected. The paralytic symptons were observed for the following I0 days, and the body wt. was measured on the 5th and 10th days. Basal diet: polished rice powder, 70%; defatted casein, 25%; McCollum & Simonds salts mixt.; cod liver oil, 2%, unsaponified substance of cod liver oil, 2%, and oryzanine powder (vitamin B_1), 1.5%. The employed oils were olive (II), soya bean (III), cocoanut (IV), sasame (V), cotton-seed (VI) and linsead (VII). Each of them (II–VII) was added to the basal diet by the amts. of 5, 10, 15%. The resistance of II or III against I. was found in 16 of 24, and 6 of 17 mice.

Unsaponified substance (III′) from III acetone insol. substance (III″) from III′ and sterins, (sistostein) from III″ were highly active.

31. The influence of fat-diet upon the skin reaction by diphtheria toxin. Tsuniji Shoji, 359–369.

Guinea pigs (200-400 g.) were fed with daily 50 g. of non-fat diet, contg. a trace of fat (about 2%). 0.1 ml. of toxin (I) was injected to the abdominal skin, and the effect of added oil in diet was represented by the min. dilution of I to result the positive skin reaction: (1) Soya bean oil (I), daily, 3g. for 10 days resulted 1/200 D.l.m. (control, 1/600 D.l.m.). (2) Acetone-sol. (II) and-insol. (III) fractions were isolated from I. The injection of 70 mg. of II and 30mg. of III dissolved in 1 ml. of olive oil per day for 3 days the results were 1/800, and 1/400 D.l. m., respectively. (3) From III, sterin (IV) was isolated by digitonin. The injection of IV (20 mg. in 1 ml. olive oil/day) and of digitonin-unbound substance (7-8 mg. in 1 ml. olive oil/day) for 3 days, showed the results of 1/400 and 1/800 D.l.m., respectively. (4) The injection of various sterine (each 17.5 mg. in 1 ml. of clive oil/day) for 4 days, gave the following results: sistosterin, 1/400: ergosterin, 1/500; cholesterin, 1/600; Ac-and Bz-cholesterin ester $<$1/800.

32. On the influence of the injected hydnocarpic oil upon the water contents of rabbit organs. Ryoji Ozawa, 370-371.

On the 5th and 10th days after the injection of hydnocarpic oil (I) to rabbits, the water contents of organs were detd. To the controll group, olive oil was injected. By I-injection, the water content was increased in liver, kidney, and brain, while the simultaneous injection of vitamin B_1 and I, redueed the water contents in the above 3 organe, approx. to those of the control.

33. A new method for the determination of diastase in urine. Saburo Setoyama, 372–378.

Urine diastase value was detd. by (1) the reducing powers (Hagedron-Jensen's method), and by (2) the amts. of glycogen (orcin-H_2SO_4 reaction), before and after the incubation (pH 6.8, 38°, 2 hrs.) of glycogen with urine. In the latter procedure, thc products from glycogen by diastase, (maltose and glucose) should be destroyed by heating at 1000° in a bath for 15 min. in the presence of 30% KOH, before colorimetry.

34. The changes of iodine number of liver lipids after the administration of cod-liver oil. Kiyosi Oikawa, 379-382.

The adult mice fed with normal diet. were administered with 10% of oil by food. Just 1 day after the administration, livers of 6-10 mice were cut out, well mixed, and the I_2-number of phospholipid (I) or fatty acid (II) fraction was detd. by Wijs method. (1) Cod liver oil (III), and linseed oil (IV), resulted the rise in I_2-number: in I, normal 77.6; III, 110.8; IV, 115.9, and in II normal 145.2; III, 175.1; IV, 160.9. (2) Fatty acids (III′, IV′) and unsaponifidd substancds (III″, IV″), isolated from III and IV, restectively: in I fraction, III′+III″, 174.9; IV′+IV″, 165.2; III′+IV″, 175.7; IV+III″, 157.9, and in II fraction, III′+III′, 143.9; IV′+IV″, 129.7; III′+IV″, 156.8; IV+III″, 129.7. (3) The contents of unsatrd. fatty acids (III‴, IV‴) in III and IV were 27.5 and 10.6%, corresponding to be 363 and 223 of I_2-number, respectively. The administration of III‴ and IV‴ resulted the rise in I_2-number: in I fraction, III‴, 178 2; IV‴, 149 3, and in II fraction, III‴, 138.8; IV‴, 87.3. Thus the rise in I_2-number after the administration of oils, is considered to be due to III′ or IV′, particularly, to III‴ or IV‴ contained in III or IV,

35. Studieson the constitution of nucleic acid (Metabolism of nucleic acid). XI. The basic capacity of thymus nucleic acid. Masakazu Tsuji, 383-390.

In order to detn. the constitution of thymus nucleic acid (I), the 1 ry and 2 ry OH-groups of phosphoric acid in I were titrated with NaOH in the presence of phenolphthalene, and the amts. of P, and N, were also analyzed. I prepd. from cow spleen or cod sperm, was found to be tetrabasic acid (3.84-3.92 equiv. of OH-group per mole of OH-group per mole of I), which may support Makino's ring-form of I.

36. Ibid. XII. The basic capacity of yeast nucleic acid. ibid., 391-397.

Yeast nucleic acid (II) was also found to be tetrabasic acid (3.89-4.00 equiv. of OH-group per mole of II), which may be an evidence for the ring-form.

37. Ibid. XIII. Alkaloid salts of nucleic acid. ibid., 398-403.

To the purified I or II, brucin (III) or stryquinine (IV) was added. The amts. of III or IV added, were more than 5 moles per mole of I or of II. From the ppt. of I or II with III or IV, the combined amts. of III or IV was analyzed, and calcd. to be 4 moles per mole of I or II which may be also an evidence for the ring-form.

38. Ibid. XIV. On the β-nucleic acid. ibid., 404-411.

Pancreatin (V) was used as nucleogenase to catalyze the depolymerization of α-nucleic acid (VI) to β-nucleic acid (VII). V caused no liberation of phophoric acid, purine, and pyrimidine. The agglutination of IV in 5 % water soln. was observed, but not the ease with VII. After VI of cow spleen had been incubated with V at 37°, pH 6.8, for 24 hrs., the filtrate showed no rise in basic capacity. VII was prepd. from this filtrate and found to be tetrabasic. The action of V. caused the deerease of viscosity of VI to that of II, which may be an evidence for the approx. same mol. wt. of VII as that of II. From these observations, the author explains that the ribose of VI is not ring-form, (<1.4> ribose) as it is the case with VII: but the remaining OH-groups attached to C atoms are combining each other, the latter ester-bonds being attacked by I.

39. Ibid. XV. Thymus nucleic acid from cod sperm. ibid., 412-413.

I from cod sperm showed the agglutination neither in 5% water soln., nor even in the addn. of 20% acetic acid. VII isolated after the action of V, was found to be tetrabasic and to be almost the same viscosimetric value as that of I from cow spleen.

40. The deficiency of vitamins in the growing period, and its influence upon the successive growth. Michiaki Hayashi, 417-421.

After a period of the deficiency of vitamin A or B_1 or B_2, in growing mice, the administration of each deficient vitamin brought about the recovery of the growth of body wt., or of tail-length, which was slower in male than in female. B_2 showed the greatest influence upon the growth of tail, and B_1 seemed to take the indirect effet on the growth by promoting the appetite.

41. Studies on the early detection of avitaminosis B_1. Ichiro Nakagawa, Chikako Oguchi, & Fusayo Fukuyama, 422-429.

Five healthy women were administered with vitamin B_1-deficient diet (I) (ca. 150 γ/day) for 60 days, subsequently with the B_1-added diet (II) (1 mg/day) for 20 days. (I contained enough amts. of protein, calories, and daily amts. of 15 mg. $FeCl_3$, 0.5 table spoon of cod liver oil (vitamin A), 1 mg. of vitamin B_2, and 50 mg. of vitamin C.)

It was found to be avarlable, for the diagnosis of latent deficiency to detn. the reduced amts. of pyruvate and B_1 in blood, which may be recovered to the normal values by B_1 addn.; while the other clinical symptoms and findings had not yet appeared at this latent period.

42. Histaminase in pregnancy. Yoshiwo Tsumuji, 429-437.

Histaminase (I) activity was measured as follows: 2 ml. of 0.01 *M* of histamine (II) soln., specimen contg. I, and 1 ml. of 200 mg % indigocarmine (III) are mixed, aerated for 30 min., and incubated at 38°, pH 7.2, for 20 hrs. The H_2O_2 formed from II by I, decolorizes III, and the remaining amts. of III is detd. by photometry (S

61). The exts. with $M/15$ phospate buffer (pH 7.2) of acetone powder of placenta (IV), of urine (V), and heat-treated serum (VI) (56°, 60 min.), were used as I sources. VI should be deproteinized with CCl_3COOH before phctometry. I was not fonnd in the inpregnant blood serum, in nable serum, and in any urine. Activity of I of VI, began to inerease with the beginning of pregnancy, to the max., at the end of pregnancy, and to decreased to zero on the 5th days later after partirition. I was found in IV of the 4th month pregnancy, higher in the normal partrition (10th month), but nct in IV of vesicular mole.

43. On the decolorization of indigocarmine in the experiments of histaminase. Yoshio Tsumuji, 437-442.

Histaminase (I) prepd. from acetone powder of placenta or pig kidney showed its action on diamine (ethylene diamine, cadaverine) as well as on proper histamine; but neicher on monoamine nor on amino acids. Activity of I was dependent upon the condition of gas phase in expl. vessel: aeration > no aeration > vacuun. The decolorization of indigocarmine by H_2O_2 was accelerated, remarkably by 0.005 $M/1$ Cu^{++} and by dil. blood $\left(\frac{1}{1000} - \frac{1}{2000}\right)$, slightly by 0.005 M Fe^{+++}, and extremely by the mixt. of Cu^{++} and blood.

44. Metabolism of ascorbic acid from a standpoint of the minimum requirements. Shizue Oda, 442-446.

Seven women (44-57 kg.) were administered with 200 mg. of vitamin C (I) per day, until I had been satrd. in tissues, which was judged by the amts. of I in blood and urine. Subsequently, the administration of I was decreased to 10-100 mg for 6 days, followed by 200 mg. on the 7th day. The amts. of I in blood and urine were decreased, when less than 80-100 mg of I was administered for 6 days. The max. requirements of I was therefore, found to be 80-100 mg. per day. When daily 30 mg of I had been administered for 2 months, it happened no appreciable change in amts. of I. Daily 20 mg. of I. caused a remarkable decrease of I in the latter half period. Thus, the min. amts. of I was found to be 20-30. mg. per day.

45. Quantitative determination of vitamin A. Akiji Fujita, & Junya Teruuchi. 447-450.

To 1-5g. of fine-pulped tissue in a glass-stopped tube, add 15 ml. of 5% KOH-EtOH, aerate with H_2 gas, warm at 75° for 20-30 min., cool rapidly under tap water, add 30 ml. of water and 5-15 ml. of benzol, aerate with H_2 gas again, collect the upper benzol-layer, wash twice with 30 ml. of 60% MeOH, subsequently twice with 30 ml. of water.——these washings should be done by filling with CO_2 or H_2, and in cold. Evaporate the benzol soln. at 30-40° *in vacuun*, dissolve the residue rapidly with 2 ml. of $CHCl_3$, under H_2 gas. To 2 ml. of this $CHCl_3$ soln., add 3 ml. of $SbCl_3$ reag. and just 5 sec. later, run photometry (S 61).

46. Quantitative determination of provitamin A. ibid., 451-455.

Well ground tissue was mixed with

15 ml. of 5% KOH-EtOH, let stand over night, ext. with 5-15 ml. of benzol, wash with 60% MeOH, then with water, evaporate the benzol layer, dissolve the residue in petroleum ether, pour into a absorption tube of alumina, drive off carotene (α, and β) with petroleum ether-benzol (1 : 1). and collect for the further analysis. The upper layer of cryptoxanthine is pushed out and dissolved in the mixt. of each 3 ml. of MeOH, of petroleum ether, and of water. The amts. of carotene or cryptoxanthine is detd. by photometry (S 47). Partitions of α-and β-carotene, vitamin A, and provitamin A are performed by various solvents.

47. Vitamin contents in foods XXI. Akiji Fujita, Junya Teruuchi, Ken Kihara, Yanako Yuasa, & Yoshie Saito. 455-457.

The contents of vitamin A B_1, B_2, and C were measured over 100 kinds of foods.

48. Distribution of vitrmin B_2 in plants and animals. II Sasuke Ōkubo, 458-461.

49. An improved method for the determination of vitamin B_2, and the comparison with the enzymatic method. Akiji Fujita, & Ryuko Yuasa, 463-464.

An improvement exists in warming tissue prior to extn., which gives higher B_2 values than the old method. The results with this improved method give a considerably good agreement with those by phosphatase method.

50. Quantitative determination of free and ester-formed vitamin B_2. Sachiko Iinuma, 464-467.

Two methods were compared in detg. B_2 in 55 of plant and 19 of animal foods: (1) Warm (at 70-80°, for 15 min.), grind, actidify (25 ml. of 1 N H_2SO_4), warm, and ext.; (2) grind, acidify, warm, and ext. The former yielded generally rather higher values than the latter. Ester-form was, mostly, 20-30% of the total.

51. On the distribution of free- and ester-formed vitamin B_1 in plant and animal tissues, and in their manufactured foods. Ken Haraki, 468-470.

52. The contents of vitamins in foods by the standard methods. Akiji Fujita, Junya Teruuchi, Ken Haraki, Ryuko Yuasa, & Yoshie Saitō, 471-478.

The contents of vitamin A, B_1, B_2, and C in more than 100 species of vagetable foods were measured with authors standard methods.

ABSTRACT

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1. The contents of vitamins in animal foods determined by the standardized methods. Akiji Fujita, Junya Teruuchi, Ken Haraki, Ryuko Yuasa & Yoshie Saito. p. 1–6, cf., 18, 470 (1946).

2. Chemical studies on antigen. Shiro Fujimura p. 7–11.

Hemolysine (I) was obtained from serum of rabbits immunized with erythrocytes. By Na_2SO_4 fractionation, I was found only in pseudoglobulin fraction (II) of the serum. Immunization caused the increase of III. Both by the action of trypsin and by heat (75°, 15 min.). III was inactivated. Thus, I was consdered to be of protein nature, but, II of immunized serum showed the same chemical compositions as those of II of normal serum. The latter did not aot as antigen against normal rabbits.

3. On the tolerance of vitamin B_2 Sasuke Okubo. p. 11–14.

The author was administered with vitamin B_2 ($V.B_2$) and 24 hrs. urine was collected for 3 days to examine the excretion of $V.B_2$. The amts. of urinary excretion of $V.B_2$ was corrected by those on 2nd and 3rd days: 3200 γ, and 1600 γ of $V.B_2$ caused 85% and 45% excretion, respectively; subcut. iniection of 950 γ of $V.B_2$ resulted 44% excretion; 1700 γ of adsol-absorbed $V.B_2$ seemed to cause the lower absorption and the delayed excretion (38% excretion).

4. On the components of bile juice of *Nutria*. Taro Kazuno, & Tsuniharu Takuma, 14–25.

From the bile juice of *Myocaster Coypus Mol.*, glyco-3-oxy-7-ketocholic and ursodesoxycholic acids were isolated and identified.

5. On the metabolism of hexose in cancer tissue. I. The intermediates of hexose by survived cancer tissue. Kozo Hara, p. 25–34.

A piece of *carcinoma simplex* tissue was subcut. transplanted to mouce and 2–4 weeks later, 0.2–0.5 g. of the grown cancer tissue (I) was cut out for further analyses. As the control, 0.2–0.5 g. of normal mouce liver (II) was used. I or II was analyzed for the contents of lactate (III), pyruvate (IV), and pentose (V), after deproteinization. The following results were obtained: I showed higher values of III, V, and remarkably, of IV, than II did; the incubation of II, before deproteinization, at pH 7.5, 37°, for 3–10 hrs., caused much increases of III and V, and, rather, a slight decrease of IV, while the incubation of I rasulted no remarkable change of III, IV, and V; when ground I or II was incubated with 10 ml. of 4% glucose at 37°, pH 7.5, for 3–5 hrs., it occurred the re-

markable increases of III and V, which were greater in I than in II, while IV was coasiderably increased in I, but rather decreased in II.

6. Ibid. II. The influence of vitamin B_1 upon the intermediates of glucose in cancer tissue. Kozo Hara, p. 35-39.

Three hrs. after the injection of 0.1–0.5 mg. of vitamin B_2 (V.B_1) to the surrounded parts of the grown I, 0.2–0.5 mg. of I was taken out for the analyses of III, IV, and V: the injection of V.B_1 caused 41.29, and 28 59 % decreases of III and IV, respectively; the incubation of I with 10 ml. of 4% glucose resulted very little changes of III, IV, and V, both in I and in II.

7. Ibid. III. The influence of the simultaneous injection of vitamin B_1 and phosphate upon the intermediates of glucose in cancer tissue. Kozo Hara, p. 39-42.

Each 0.1 ml. of the soln., dissolving 1.0379 g. of V.B_1 in 100 ml. of $M/15$ phosphate buffer at pH 7.0 was injected to cancer tissue, 0.2-0.5 mg. of which was, 3 hrs. later, cut out for the analyses of III, IV, and V. The greater decreases of III, and IV were observed in the injected group than in the non-injected group

8. The components of bile juice of *Hippopotamus ampyhibus*. L. and of *Ursus thibetanus japonicus* Schleger. Kozo Kara, p. 42-45.

Cholic and chenoxydesocholic acids were found in the bile juice of both animals.

9. The influence of thymus gland extracts on the growth of the transplanted cancer of mice. Kozo Hara, p 45-61.

Thymus glands of new-born calves (3-7 days) were extd. with 5 vol. of 60% EtOH for 24 hrs. at room temp., condenced *in vacuo* at 30-40°, deproteinized with 5% colloidal iron soln. and fractionated by 10 % $CuSO_4$ and 10 % Na_2WO_4-H_3PO_4-solns. into mono-amino (I)- and diamino (II)-fractions. 0.5136 g./dl ash soln (II) of the gland was prepd. To the tumor-bearing mice, 5 days after the transplantation, each 0.05 ml. of I, II, or III was subcut. injected every two days, and the growth of tumor was examined by measuring the palpable lengths and the weights of tumor, on the 25th day.

The growth of tumor was inhibited both by II and by III, but not influenced by I.; the formation of tumor by transplantation was promoted by I, and, to a certain degree, by III, but not by II.; I seemed to increase the mortality of tumor-bearing mice; the body wt. was not influenced by both I, II, and III.

10. On the bile juice of *Bulbus bubalis*. Shunii Mizuhara & Kenii Matsumoto, p. 61-63.

From 150 g. of the bile juice of *Bulbus bubalis*, 9.3 g. of cholic, 2.1 g. of desoxycholic, 0.1 g. of chenoxydescholic, and 0.2 g. of stearic acids were isolated.

11. On the quantitative determination of arginine. Tsutomu Sekine, p. 63-65.

Ten ml. of the mixt. containing each 2 ml. of arginase, $M/10$ arginine—HCl

soln. and buffer (pH 6~9) was incubated at 37°. To 4 ml. of this mixt., add 1 ml. of 10% Pb-subacetate, and 1 ml. of 10% $NaNO_3$, and filter. To 3 ml. of the filtrate, add 1 ml. of 1.5% H_2SO_4, and filter. To 1 ml. of this filtrate, add 1 ml. of 3% flavin soln., by which the crystal of arginine-flavianate (I) was pptd, followed by washing I with alcohol. I was dissolved in alkaline soln., and reduced by $SnCl_2$ in the presence of Na, K-tartarate, for the colorimetry.

12. Studies on hexosidases. VII. On the inhibition of β-hexosidase by β-hexosides. Koichi Kobayashi, & Kiyohide Okazaki, p. 65–70, cf., 18, 41 (1944); J. Biochem., 32, 91, 107 (1940).

β-Hexosidase activity was detd. by the colorimetry of *p*-nitrophenol liberated from *p*-nitrophenol-β-hexoside as substrate. β-Hexosides of *o*-(I), *m*-(II), and *p*-(III) cresol, and of phenol (IV) demonstrated the following inhibitions: (1) emulsin-type, (a) β-galactosidase (emulsin, *Escherichia coli*), and (b) β-glucosidase (emulsin, *Prot. vulgaris*), III > II > IV > I; (2) taka type, (a) β-glncosidase (Takadiastase, durra), IV > II > III > I and (b) β-galactosidase (Takadiastase, pig spleen), II > III > IV > I.

13. Ibid. VIII. On the affinity of β-hexosidase. Kiyohide Okazaki, p. 71–77.

From kinetical date of β-glucosidase, Michaelis const. (K_m) and the affinity-consts (II) were detd.: (1) taka-type (durra), $pK_m = 3.7$, II = 5000 cal.: (2) emulsin-type (emulsin), $pK_m = 2.6$, II = 460 eal.; (3) takatype (Takadiastase) gave pS-activity curve of Bell's form, which may be interpreted by assuming two affinity groups with $pK_m' = 3.7$, and $pK_m'' = 2.6$.

14. On the hydrolysis of *l* (+)–arginine by *Streptococcus faecalis*. Tsutomu Sekine, p. 79–85.

Strept. faecalis isolated from infant feces, was cultured in 1% arginine-agar, from which suspension (I) in sterilized water was prepd. I showed to split *l* (+)–arginine (II) into NH_3 and citrullin (III), the latter (III) being further hydrolyzed to NH_3 and ornithive by I. No inhibition of Mn^{++}, Fe^{++}, KCN, glutathione, and cysteine were observed. The opt. pH of I on II or III was found to be 7.0. I did not act on *d*-arginine, *l*-arginic acid, glycocyamine, and hydantoin.

The suspension of *Strept. faecalis* cultured in glucose-bouillon, showed the decarboxylation of III at pH 4, but no NH_3 liberation.

15. On the specifity of hetero-arginase. Gaijiro Kobayashi, p. 85-91.

Heteroarginase (I) prepd. by extg. rabbit intestinal mucosa with 10% sucrose soln. was found to act on only *d* (–)-arginine, but not on *l* (+)-arginine. Among other ω-guanido-fatty acids, δ-guanidovaleric and γ-guanidobutyric acids were hydrolyzed by I, accompanying urea formation.

16. Metabolism of *l* (+) arginine. Gaijiro Kobayashi, p. 92–93.

A review on the metabolic pathways of arginine to putrescin.

17. Supplemeutary study on Rotter's skin reaction for avitaminosis C. Takanori Date, p. 93–102.

This skin reaction was proved to be reliable as the indicator of vitamin C contents in animal body, from expts. with scorbutic guinea pigs; the time of this reaction was found to be less than 6 min. for the normal and more than 10 min. for the deficient.

18. The application of Rotter's skin reaction to peoples in Tokyo city and in neighbor countries. Takanori Date, p. 103–104.

19. Studies on β-hexosidases. IX. On the mechanism of enzyme activity. Akira Kaya, p. 105–111, cf., **18**, 41; **19**, 65, 71 (1947); J. Biochem., **35**, 39, 293 (1942).

β-Hexosidase activity was kinetically interpreted by use of the data in the previous studies.

20. The determination of co-phosphosomonesterase. Koichi Kobayashi, p. 112–118.

Co-(I) and apo-(II) phosphomonoesterase were prepd. from pig kidney, or rice bran, by Kumaki's method (J. Biochem., **33**, 277 (1941)). The activity of holo-enzyme (III), synthesized with I and II, was detd. against *p*-nitrophenol-phosphate at 37° and pH 9 in the presence of 1 ml. of $M/20$ $MgCl_2$. The acitvity of III was proportionally increased, to a limit, by the addn. of I to a const. amt. of II. Thus the amt. of I could be detd. from the activity of III. Urine showed to contain I, the amts of which was increased by removing phosphate with baryta and Na_2CO_3, MgO, or baryta and active charcoal.

21. The reaction of amino acids, peptides, and related substances with sugars. III. The reaction with formaldehyde. Shimpei Araya, p. **11** p. 117–122, cf., N. Shiga, J. Biochem., **25**, 607 (1837); **27**, 103 (1938).

Either Amino acid, or peptide, or related substances, and formaldehyde (I) was dissolved in 1 l. of buffer, contg. 45 g. of KH_2PO_4 and 95 g. of citric acid, and pH was adjusted with 4 N NaOH. Each mixt. was incubated at 22–24°, and an aliquot was pipetted out, from time to time, to detn. the combined amts. of 1 from remaining amts. of $-NH_2$ with van Slyke's method. The combined amts. of I (0.025 M) with glycine (0.016 M) was "amino acid type" (pH 9> 8> 7); of I (0.025 M) with glycylglycine (0.016 M) was initially pH 8> 7> 9, which changed into "amino acid type" after 1 hr.; of I (0.35 M) with casein was also "amino acid type"; of I (0.36 M) with acetamide (4.4 M) was detd. from the uncombined amts. of I by using Cajori's iodometry, which indicated more combination of I in neutral soln. than in alkaline or acid sides. The type of the combination of I was shown to be analogous to that of glucose the former being, however, far faster than the latter. From these observations, formol titration was discussed from view-point of amphoretic ions.

22. Ibid., IV. The combination of pepton or various proteins with glucose. Shimpei Araya, p. 123-127.

The combined amts. of glucose (I) was detd. from remaining amts, of $-NH_2$ with Van Slyke's method, in which amino acid- or peptide-N, and ε-amino acid-N were measured by shaking for 5-10, and 20-30 min., respectively. The combination of I with 1% *Witte* pepton was found to be pH 7> 9, and pH, 9> 7, by 5, and 30 min. shakings, respectively; the features of the combination of I with cow blood globin and horse serum casien or gelatin, by 5-10 min. shaking were found to be "amino acid type".

23. Co-phosphomonoesterase. Koichi Kobayashi, p. 129-133.

Various compds, including amino acids and amines, were examined for the co-phosphomonesterase activities by the method in the previous reports. (this Abstr., **19**, 112 (1947)). The co-enzyme action was found to be greater in the following order: of 20 amino acids, histidine, ≫ aspartic acid, tyrosine, diaminosuccinic acid, glycine > glycylglycine, cysteine, phenylanine; of 6 amines, only histamine; benzoyl- histidine, glycine, and glutamic acid were inactive. No optical specifity was observed with *d*- and *l*-histidine.

24. Biochemical Studies on phospholipids. III. Sphingosine. cf.. this Abstr., **18**, 151 (1944). Kokichi Ohno. p. 133-137.

From beef brain, sphingosine (I) was isolated, and dihydrosphingosine (II) was prepd. from I. Palmitinaldehyde was identified as the oxidation product of II with Pb-tetracetate (III). I was found to be oxidized by III to *trans*-hexadencen (2, 3)-al, which was further oxidized by Ag_2O to hexadecen (2, 3)-acid. The structure of I was thus considered to have either NH_2 at C_1, OH at C_2, and OH at C_3, or OH at C_1, NH_2 at C_2, and OH at C_3, and *trans* formed double bond between C_4 and C_5.

25. The reaction of amino acids, peptides, and related substances. V. The influence of solvents in relation to the dissociation constant of amino group. Shimpei Araya, p. 137-142.

The combination of glucose (I) with amino acid or peptide, dissolved in 9.5 *M* acetamide (II) or in dioxane (III) was examined, and classified in 3 types. (1) tripeptide type: glycine in II, and in III (50%); (2) amino acid type; methylamine in II, and in III (85%): (3) peptide type: cystine or cysteine. K_B (the diss. const. of amino group) was calcd. to be 100, 10, and 1×10^{-7} *M*, for amino acid, dipeptide, and tripeptide, respectively.

26. ibid. VI. The reaction of amino acid with various sugars. Shimpei Araya, p. 143-145.

The reaction of 0.018 *M* glycine (I) with pentose or hexose was examined. The combined amts. of each sugar with I were greater in the order of mannose> galactose> glucose; arabinose> xylose; and those with II were galactose> glucose.

27. The fractionating determination of aromatic hydroxy- and amino- compounds. I, A micro-estimation with dibromoaminophenol reagent. Yasuyuki Shishikura, p. 145–149.

For the detn. of phenol, 2, 6-dibromo-4-aminophenol (DBAP) was used. To 1 ml. of a specimen contg. M/2000–M/200000 I, in a calibrated test-tube, add 1 ml. of 3% borax, adiusted pH 9 with dil.-NaOH or –HCl, add 0.2 ml. of 10% $K_3Fe(CN)_6$, and 0 2 ml. of 0.1% DBAP. Incubate in a water-bath at 37° for 5 min. After cooling, add dist. water to the 10 ml.-mark, and let stand for 10 min. The developed color should be detd. within 60 min., with photometer (S 61). For the detn. of salicyluric acid (II), anthranilic acid (III), or aniline (IV), add 1 ml. of 2% NaOH after the incubation——by this addn. the color of II, III, or IV, with DBAP disappears——, remove the interfering colored substances with butanol, and estimate the color intensity with a photometer (S 61). The following ratis of C (mg./dl.)/E were obtained.: I, 4.012; II, 6.8 1; III, 15 86: IV, 11 63; V, 16.59.

28. Quantitative determination of nicotinic acid in tissues. Kazuwo Kawashima, p. 149–159.

(1) Hydrolysis: the animal tissues with 4 ml. of conc. HCl at 100° for 60 min. Neutralize, and dilute to 25 ml. with dist. water. (2) Protein removal: 10 ml. of the hydrolysate+2 ml. of 40% Pb-acetate+ 1 ml. of Na_2S (40% NaOH, satrd. with. H_2S). (3) Colorimetry: 4 ml. of deproteinized filtrate was adjusted to pH 6. If turbidity occurs, centrifuge off after standing (5–6 hrs). To 1 ml. of this faint yellowish soln. (I), add 1 ml. of BrCN soln., and 1 ml. of M/3 phosphate buffer (pH 6.0), heat at 100° for 10 min., add 1 ml. of 4% anilinealcohol soln. The developed color should be measured within 3 min. by use of photometer with S 47. The blank (1.0 ml. of I), and the control (1.0 ml. of I + 0.2 ml. of 100 mg. % nicotinic acid) should be carried out to obtain the corrected value by caculation.

29. On the quinoline- and acridine- dehydrogenases. Yukichi Hashimoto, p. 159–163.

Various quinoline- and acridine-derivatives were examined for the oxidation with 10% water exts. of rabbit liver brei (I) by use of Thunberg tube at pH 7.0, and 37°, in which each 1 ml. of I, 0.005% methylenblue (MB), and of 0.01% each derivative had been mixed. Quinoline (II), and acridine (III) were oxidized with 7, and 8 min. of the decolosization time of MB., respectively. 8-Me-, 8-OH-, and N-alkylhalogen-derivattves of II prolonged the decolorization time. Compds of IV at C_8 atom, were no more oxidized. O_2 uptake showed the liberation of 2 atoms of H per 1 mol. of III or IV oxidation. The loss of acitivity of I, by washing in slice state, was restored by the addn. of flavin co-enzyme, prepd. from the filtrate of boiled exts. of rabbit liver.